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VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 49/00, 47/48	A3	(11) International Publication Number: **·WO 98/18498**
		(43) International Publication Date: 7 May 1998 (07.05.98)

(21) International Application Number: PCT/GB97/02958

(22) International Filing Date: 28 October 1997 (28.10.97)

(30) Priority Data:
9622364.9	28 October 1996 (28.10.96)	GB
9622367.2	28 October 1996 (28.10.96)	GB
9622366.4	28 October 1996 (28.10.96)	GB
9700698.5	15 January 1997 (15.01.97)	GB
9700699.3	15 January 1997 (15.01.97)	GB
9708265.5	24 April 1997 (24.04.97)	GB
9711842.6	6 June 1997 (06.06.97)	GB
9711844.2	6 June 1997 (06.06.97)	GB

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications
US	60/050,941 (CIP)
Filed on	6 June 1997 (06.06.97)
US	60/049,265 (CIP)
Filed on	6 June 1997 (06.06.97)
US	60/049,264 (CIP)
Filed on	6 June 1997 (06.06.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
16 July 1998 (16.07.98)

(54) Title: IMPROVEMENTS IN OR RELATING TO DIAGNOSTIC/THERAPEUTIC AGENTS

(57) Abstract

Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generated material, in which the reporter is coupled or linked to one or more non-bioactive vectors.

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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Published
Without international search report and to be republished upon receipt of that report.

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(54) Title: IMPROVEMENTS IN OR RELATING TO DIAGNOSTIC/THERAPEUTIC AGENTS

(57) Abstract

Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generated material, in which the reporter is coupled or linked to one or more non-bioactive vectors.

Improvements in or relating to diagnostic/therapeutic agents

5　　　This invention relates to diagnostic and/or
therapeutically active agents, more particularly to
diagnostic and/or therapeutically active agents
incorporating moieties which interact with or have
affinity for sites and/or structures within the body so

10　　that diagnostic imaging and/or therapy of particular
locations within the body may be enhanced. Of
particular interest are diagnostic agents for use in
ultrasound imaging, which are hereinafter referred to as
targeted ultrasound contrast agents.

15　　　It is well known that ultrasound imaging comprises
a potentially valuable diagnostic tool, for example in
studies of the vascular system, particularly in
cardiography, and of tissue microvasculature. A variety
of contrast agents has been proposed to enhance the

20　　acoustic images so obtained, including suspensions of
solid particles, emulsified liquid droplets, gas bubbles
and encapsulated gases or liquids. It is generally
accepted that low density contrast agents which are
easily compressible are particularly efficient in terms

25　　of the acoustic backscatter they generate, and
considerable interest has therefore been shown in the
preparation of gas-containing and gas-generating
systems.

　　　Gas-containing contrast media are also known to be
30　　effective in magnetic resonance (MR) imaging, e.g. as
susceptibility contrast agents which will act to reduce
MR signal intensity. Oxygen-containing contrast media
also represent potentially useful paramagnetic MR
contrast agents.

35　　　Furthermore, in the field of X-ray imaging it has
been observed that gases such as carbon dioxide may be
used as negative oral contrast agents or intravascular

contrast agents.

The use of radioactive gases, e.g. radioactive isotopes of inert gases such as xenon, has also been proposed in scintigraphy, for example for blood pool
5   imaging.

Targeted ultrasound contrast agents may be regarded as comprising (i) a reporter moiety capable of interacting with ultrasound irradiation to generate a detectable signal; (ii) one or more vectors having
10   affinity for particular target sites and/or structures within the body, e.g. for specific cells or areas of pathology; and (iii) one or more linkers connecting said reporter and vector(s), in the event that these are not directly joined.
15   The molecules and/or structure to which the agent is intended to bind will hereinafter be referred to as the target. In order to obtain specific imaging of or a therapeutic effect at a selected region/structure in the body the target must be present and available in this
20   region/structure. Ideally it will be expressed only in the region of interest, but usually will also be present at other locations in the body, creating possible background problems. The target may either be a defined molecular species (i.e. a target molecule) or an unknown
25   molecule or more complex structure (i.e. a target structure) which is present in the area to be imaged and/or treated, and is able to bind specifically or selectively to a given vector molecule.

The vector is attached or linked to the reporter
30   moiety in order to bind these moieties to the region/structure to be imaged and/or treated. The vector may bind specifically to a chosen target, or it may bind only selectively, having affinty also for a limited number of other molecules/structures, again
35   creating possible background problems.

There is a limited body of prior art relating to targeted ultrasound contrast agents. Thus, for example,

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US-A-5531980 is directed to systems in which the
reporter comprises an aqueous suspension of air or gas
microbubbles stabilised by one or more film-forming
surfactants present at least partially in lamellar or
5   laminar form, said surfactant(s) being bound to one or
more vectors comprising "bioactive species designed for
specific targeting purposes". It is stated that the
microbubbles are not directly encapsulated by surfactant
material but rather that this is incorporated in liquid-
10   filled liposomes which stabilise the microbubbles. It
will be appreciated that lamellar or laminar surfactant
material such as phospholipids present in such liposomes
will inevitably be present in the form of one or more
lipid bilayers with the lipophilic tails "back-to-back"
15   and the hydrophilic heads both inside and outside (see
e.g. Schneider, M. on "Liposomes as drug carriers: 10
years of research" in *Drug targeting, Nyon, Switzerland,
3-5 October 1984*, Buri, P. and Gumma, A. (Ed), Elsevier,
Amsterdam 1984).
20   EP-A-0727225 describes targeted ultrasound
contrast agents in which the reporter comprises a
chemical having a sufficient vapour pressure such that a
proportion of it is a gas at the body temperature of the
subject. This chemical is associated with a surfactant
25   or albumin carrier which includes a protein-, peptide-
or carbohydrate-based cell adhesion molecule ligand as
vector. The reporter moieties in such contrast agents
correspond to the phase shift colloid systems described
in WO-A-9416739; it is now recognised that
30   administration of such phase shift colloids may lead to
generation of microbubbles which grow uncontrollably,
possibly to the extent where they cause potentially
dangerous embolisation of, for example, the myocardial
vasculature and brain (see e.g. Schwarz, *Advances in
35   Echo-Contrast [1994(3)]*, pp 48-49).
         WO-A-9320802 proposes that tissue-specific
ultrasonic image enhancement may be achieved using

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acoustically reflective oligolamellar liposomes
conjugated to tissue-specific ligands such as
antibodies, peptides, lectins etc. The liposomes are
deliberately chosen to be devoid of gas and so will not
5   have the advantageous echogenic properties of gas-based
ultrasound contrast agents. Further references to this
technology, e.g. in targeting to fibrin, thrombi and
atherosclerotic areas are found in publications by
Alkanonyuksel, H. et al. in *J. Pharm. Sci.* (1996) 85(5),
10   486-490; *J. Am. Coll. Cardiol.* (1996) 27(2) Suppl A,
298A; and *Circulation, 68 Sci. Sessions,* Anaheim 13-16
November 1995.

There is also a number of publications concerning
ultrasound contrast agents which refer in passing to
15   possible use of monoclonal antibodies as vectors without
giving significant practical detail and/or to reporters
comprising materials which may be taken up by the
reticuloendothelial system and thereby permit image
enhancement of organs such as the liver - see, for
20   example WO-A-9300933, WO-A-9401140, WO-A-9408627, WO-A-
9428874, US-A-5088499, US-A-5348016 and US-A-5469854.

The present invention is based on the finding that
gas-containing and gas-generating diagnostic and/or
therapeutic agents coupled to non-bioactive vectors are
25   particularly useful targeting agents by virtue of their
enhanced safety relative to conventional targeting
agents, which may elicit undesirable and unwanted
biological effects in the subject.

An added advantage of certain kinds of non-
30   bioactive vectors is that they may exhibit improved
targeting efficacy as compared to bioactive vectors. The
reason for this is that bioactive vectors usually will
have to compete with endogenous ligands for the same
binding site on the target molecule. In contrast non-
35   bioactive vectors will often bind to target molecules
for which endogenous ligands do not exist, or
alternatively non-bioactive vectors may bind at sites in

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a target molecule that is not involved in the biological
function of the target molecule and for which natural
ligands are not present in the body.

The term "non-bioactive" as used herein denotes
5   two kinds of substances. First, it denotes a substance
capable of interacting with or binding to a target
molecule or target structure that is not normally
involved in generating biological responses. Second, it
denotes a substance capable of interacting with or
10   binding to a given target without either giving or
inhibiting the biological response normally elicited
upon binding of a bioactive substance to the target, a
bioactive substance being one which interacts with
another molecule (i.e. a target) and generates a defined
15   and measurable biological response.

Many receptors for transport proteins, such as
receptors for transferrin or lipoproteins, do not in
themselves generate a biological response upon binding
of the ligand. Likewise a moiety carried in the protein
20   may be bioactive, but the apoprotein itself is not. In
a similar manner, many cofactors, vitamins etc. are
bioactive only after they have been carried into the
cell, that is to say, they do not elicit a biological
response simply upon binding to the carrier protein.

25   Vectors useful in accordance with the invention
may, for example, be non-bioactive *per se* or may be non-
bioactive at doses useful for diagnostic and/or
therapeutic purposes. Alternatively, a combination of
otherwise bioactive vectors may be employed in such a
30   way that a biological activity elicited by one type of
vector is exactly counterbalanced by another kind or
other kinds of vector molecules coupled to the same
agent or otherwise present in the same diagnostic and/or
therapeutic composition.

35   One advantageous embodiment of the invention is
based on the additional finding that limited adhesion to
targets is a highly useful property of diagnostic and/or

therapeutically active agents, which property may be achieved using non-bioactive vectors giving temporary retention rather than fixed adhesion to a target. Thus such agents, rather than being fixedly retained at specific sites, may for example effectively exhibit a form of retarded flow along the vascular endothelium by virtue of their transient interactions with endothelial cells. Such agents may thus become concentrated on the walls of blood vessels, in the case of ultrasound contrast agents providing enhanced echogenicity thereof relative to the bulk of the bloodstream, which is devoid of permanent structural features. They therefore may permit enhanced imaging of the capillary system, including the microvasculature, and so may facilitate distinction between normal and inadequately perfused tissue, e.g. in the heart, and may also be useful in visualising structures such as Kupffer cells, thrombi and atherosclerotic lesions or for visualising neo-vascularized and inflamed tissue areas. The present invention is also well suited to image changes occurring to normal blood vessels which are situated in areas of tissue necrosis.

Thus according to one aspect of the present invention there is provided a targetable diagnostic and/or therapeutic agent, e.g. an ultrasound contrast agent, comprising a suspension in an aqueous carrier liquid, e.g. an injectable carrier liquid, of a reporter comprising gas-containing or gas-generating material and coupled to one or more vectors, characterised in that said vector or vectors are non-bioactive.

Vectors useful in accordance with the invention include proteins which bind to cell-surface proteoglycans, e.g. so that the contrast agent becomes concentrated on cell surfaces. Such proteoglycans are large glycoproteins containing glucosaminoglycan side chains which, in many cases, are heparan sulphate.

Binding to glucosoaminoglycans has not been shown to
elicit a biological response. In accordance with the
invention one may isolate or synthesise the
proteoglycan-binding (or, where appropriate, heparan

5   sulphate-binding) part of the molecule for use as a
vector, while avoiding any biological activity
associated with other parts of the molecule.

        The use of non-bioactive monomeric or oligomeric
vectors and the use of non-bioactive peptide vectors

10   represent preferred aspects of this invention.

        A further aspect of the present invention is
for example where a vector or vectors is attached to the
reporter or included non-covalently into the reporter in
a manner where the said vector or vectors is not readily

15   exposed to the targets or receptors. Increased tissue
specificity may therefore be achieved by applying an
additional process to expose the vectors, e.g. the agent
is exposed to external ultrasound to change the
diffusibility of the moieties containing the vectors.

20   Any biocompatible gas may be present in the
reporter of contrast agents according to the invention,
the term "gas" as used herein including any substances
(including mixtures) substantially or completely in
gaseous (including vapour) form at the normal human body

25   temperature of 37°C. The gas may thus, for example,
comprise air; nitrogen; oxygen; carbon dioxide;
hydrogen; an inert gas such as helium, argon, xenon or
krypton; a sulphur fluoride such as sulphur
hexafluoride, disulphur decafluoride or

30   trifluoromethylsulphur pentafluoride; selenium
hexafluoride; an optionally halogenated silane such as
methylsilane or dimethylsilane; a low molecular weight
hydrocarbon (e.g. containing up to 7 carbon atoms), for
example an alkane such as methane, ethane, a propane, a

35   butane or a pentane, a cycloalkane such as cyclopropane,
cyclobutane or cyclopentane, an alkene such as ethylene,
propene, propadiene or a butene, or an alkyne such as

acetylene or propyne; an ether such as dimethyl ether; a
ketone; an ester; a halogenated low molecular weight
hydrocarbon (e.g. containing up to 7 carbon atoms); or a
mixture of any of the foregoing. Advantageously at
5   least some of the halogen atoms in halogenated gases are
fluorine atoms; thus biocompatible halogenated
hydrocarbon gases may, for example, be selected from
bromochlorodifluoromethane, chlorodifluoromethane,
dichlorodifluoromethane, bromotrifluoromethane,
10   chlorotrifluoromethane, chloropentafluoroethane,
dichlorotetrafluoroethane, chlorotrifluoroethylene,
fluoroethylene, ethylfluoride, 1,1-difluoroethane and
perfluorocarbons, e.g. perfluoroalkanes such as
perfluoromethane, perfluoroethane, perfluoropropanes,
15   perfluorobutanes (e.g. perfluoro-n-butane, optionally in
admixture with other isomers such as perfluoro-iso-
butane), perfluoropentanes, perfluorohexanes and
perfluoroheptanes; perfluoroalkenes such as
perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-
20   ene) and perfluorobutadiene; perfluoroalkynes such as
perfluorobut-2-yne; and perfluorocycloalkanes such as
perfluorocyclobutane, perfluoromethylcyclobutane,
perfluorodimethylcyclobutanes, perfluorotrimethyl-
cyclobutanes, perfluorocyclopentane, perfluoromethyl-
25   cyclopentane, perfluorodimethylcyclopentanes,
perfluorocyclohexane, perfluoromethylcyclohexane and
perfluorocycloheptane. Other halogenated gases include
methyl chloride, fluorinated (e.g. perfluorinated)
ketones such as perfluoroacetone and fluorinated (e.g.
30   perfluorinated) ethers such as perfluorodiethyl ether.
The use of perfluorinated gases, for example sulphur
hexafluoride and perfluorocarbons such as
perfluoropropane, perfluorobutanes and
perfluoropentanes, may be particularly advantageous in
35   view of the recognised high stability in the bloodstream
of microbubbles containing such gases.
        The reporter may be in any convenient form, for

example being any appropriate gas-containing or gas-
generating ultrasound contrast agent formulation.
Representative examples of such formulations include
microbubbles of gas stabilised (e.g. at least partially

5   encapsulated) by a coalescence-resistant surface
membrane (for example gelatin, e.g. as described in WO-
A-8002365), a filmogenic protein (for example an albumin
such as human serum albumin, e.g. as described in US-A-
4718433, US-A-4774958, US-A-4844882, EP-A-0359246, WO-A-

10   9112823, WO-A-9205806, WO-A-9217213, WO-A-9406477 or WO-
A-9501187), a polymer material (for example a synthetic
biodegradable polymer as described in EP-A-0398935, an
elastic interfacial synthetic polymer membrane as
described in EP-A-0458745, a microparticulate

15   biodegradable polyaldehyde as described in EP-A-0441468,
a microparticulate N-dicarboxylic acid derivative of a
polyamino acid - polycyclic imide as described in EP-A-
0458079, or a biodegradable polymer as described in WO-
A-9317718 or WO-A-9607434), a non-polymeric and non-

20   polymerisable wall-forming material (for example as
described in WO-A-9521631), or a surfactant (for example
a polyoxyethylene-polyoxypropylene block copolymer
surfactant such as a Pluronic, a polymer surfactant as
described in WO-A-9506518, or a film-forming surfactant

25   such as a phospholipid, e.g. as described in WO-A-
9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780 or WO-
A-9503835).

     Other useful gas-containing contrast agent
30   formulations include gas-containing solid systems, for
example microparticles (especially aggregates of
microparticles) having gas contained therewithin or
otherwise associated therewith (for example being
adsorbed on the surface thereof and/or contained within

35   voids, cavities or pores therein, e.g. as described in
EP-A-0122624, EP-A-0123235, EP-A-0365467, WO-A-9221382,
WO-A-9300930, WO-A-9313802, WO-A-9313808 or WO-A-

9313809). It will be appreciated that the echogenicity
of such microparticulate contrast agents may derive
directly from the contained/associated gas and/or from
gas (e.g. microbubbles) liberated from the solid
5   material (e.g. upon dissolution of the microparticulate
structure).

The disclosures of all of the above-described
documents relating to gas-containing contrast agent
formulations are incorporated herein by reference.

10   Gas microbubbles and other gas-containing
materials such as microparticles preferably have an
initial average size not exceeding 10 μm (e.g. of 7 μm
or less) in order to permit their free passage through
the pulmonary system following administration, e.g. by
15   intravenous injection.

Where phospholipid-containing compositions are
employed in accordance with the invention, e.g. in the
form of phospholipid-stabilised gas microbubbles,
representative examples of useful phospholipids include
20   lecithins (i.e. phosphatidylcholines), for example
natural lecithins such as egg yolk lecithin or soya bean
lecithin and synthetic or semisynthetic lecithins such
as dimyristoylphosphatidylcholine,
dipalmitoylphosphatidylcholine or
25   distearoylphosphatidylcholine; phosphatidic acids;
phosphatidylethanolamines; phosphatidylserines;
phosphatidylglycerols; phosphatidylinositols;
cardiolipins; sphingomyelins; fluorinated analogues of
any of the foregoing; mixtures of any of the foregoing
30   and mixtures with other lipids such as cholesterol. The
use of phospholipids predominantly (e.g. at least 75%)
comprising molecules individually bearing net overall
charge, e.g. negative charge, for example as in
naturally occurring (e.g. soya bean or egg yolk
35   derived), semisynthetic (e.g. partially or fully
hydrogenated) and synthetic phosphatidylserines,
phosphatidylglycerols, phosphatidylinositols,

phosphatidic acids and/or cardiolipins, may be
particularly advantageous.

Other exemplary lipids which may be used to
prepare gas-containing contrast agents include fatty
5   acids, stearic acid, palmitic acid, 2-n-hexadecylstearic
acid, oleic acid and other acid containing lipid
structures. These lipid structures are considered
particularly interesting when coupled by amide bond
formation to amino acids containing one or more amino
10   groups. The resulting lipid modified amino acids (e.g.
dipalmitoyllysine, distearoyl-2,3-diaminopropionic acid)
are considered useful precursors for the attachment of
functionalised spacer elements featuring coupling sites
for conjugation of one or more vector molecules.

15   A further extension of this invention relates to
the synthesis of lipopeptide structures comprising a
lipid reporter attached to a linker portion (e.g. PEG,
polyamino acid, alkylhalide etc) the said linker being
suitably functionalised for coupling to one or more
20   vector molecules. A particular preference is the
inclusion of a positively charged linker element (eg.
two or more lysine residues) for anchoring of the
reporter element in the microbubble through
electrostatic interaction with the negatively charged
25   membrane.

Also considered within the scope of this invention
are functionalised microbubbles carrying one or more
reactive groups for non-specific modification of a
multiplicity of receptor molecules located on cell
30   surfaces. Microbubbles comprising a thiol moiety,for
example, can bind to cell surface receptors via
disulphide exchange reactions. The reversible nature of
this covalent bond means that bubble flow can be
controlled by altering the redox environment. Similarly
35   `activated' microbubbles of membranes comprising active
esters such as N-hydroxysuccinimide esters can be used
to modify amino groups found on a multiplicity of cell

surface molecules.

Representative examples of gas-containing microparticulate materials which may be useful in accordance with the invention include carbohydrates (for
5   example hexoses such as glucose, fructose or galactose; disaccharides such as sucrose, lactose or maltose; pentoses such as arabinose, xylose or ribose; α-, β- and γ-cyclodextrins; polysaccharides such as starch, hydroxyethyl starch, amylose, amylopectin, glycogen,
10   inulin, pulullan, dextran, carboxymethyl dextran, dextran phosphate, ketodextran, aminoethyldextran, alginates, chitin, chitosan, hyaluronic acid or heparin; and sugar alcohols, including alditols such as mannitol or sorbitol), inorganic salts (e.g. sodium chloride),
15   organic salts (e.g. sodium citrate, sodium acetate or sodium tartrate), X-ray contrast agents (e.g. any of the commercially available carboxylic acid and non-ionic amide contrast agents typically containing at least one 2,4,6-triiodophenyl group having substituents such as
20   carboxyl, carbamoyl, N-alkylcarbamoyl, N-hydroxyalkylcarbamoyl, acylamino, N-alkylacylamino or acylaminomethyl at the 3- and/or 5-positions, as in metrizoic acid, diatrizoic acid, iothalamic acid, ioxaglic acid, iohexol, iopentol, iopamidol, iodixanol,
25   iopromide, metrizamide, iodipamide, meglumine iodipamide, meglumine acetrizoate and meglumine diatrizoate), and polypeptides and proteins (e.g. gelatin or albumin such as human serum albumin).

30   The reporter may be made by any convenient process, for example by making gas-containing or gas-generating formulations. Representative examples include the preparation of a suspension of gas microbubbles by contacting a surfactant with gas and mixing them in the
35   presence of an aqueous carrier, as described in WO 9115244; or by atomising a solution or dispersion of a wall-forming material in the presence of a gas in order

to obtain hollow microcapsules, as described in EP
512693A1; preparation of solid microspheres by a double
emulsion process, as described in US 5648095; or a
process for forming hollow microcapsules by spray-drying
5   as described in EP 681843A2; or preparing gas-filled
liposomes by shaking an aqueous solution comprising a
lipid in the presence of a gas as described in US
5469854.

A suitable process for attachment of the desired
10   vector to the reporter comprises a surface modification
of the preformed reporter with a suitable linker
employing reactive groups on the surface of both the
reporter and vector. It may be particularly advantageous
physically to mix the reporter material with the vector-
15   containing substance at any step of the process. Such a
process will result in incorporation or an attachment of
the vector to the reporter. An optional process step may
remove the excess of vector not bound to the reporter by
washing the gas-containing particles following
20   separation, by for example, floatation. A preferred
aspect is the use of lipopeptide structures
incorporating functional groups such as thiol, maleimide
biotin etc. which can be premixed if desired with other
reporter molecules before formation of gas-containing
25   agents. The attachment of vector molecules may be
carried out using the linker reagents listed below.

Coupling of a reporter unit to the desired vectors
may be achieved by covalent or non-covalent means,
usually involving interaction with one or more
30   functional groups located on the reporter and/or
vectors. Examples of chemically reactive functional
groups which may be employed for this purpose include
amino, hydroxyl, sulfhydryl, carboxyl, and carbonyl
groups, as well as carbohydrate groups, vicinal diols,
35   thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyl,
imidazolyl and phenolic groups.

Covalent coupling of reporter and vectors may

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therefore be effected using linking agents containing
reactive moities capable of reaction with such
functional groups. Examples of reactive moieties
capable of reaction with sulfhydryl groups include α-
5   haloacetyl compounds of the type X-CH$_2$CO- (where X=Br,
Cl or I), which show particular reactivity for
sulfhydryl groups but which can also be used to modify
imidazolyl, thioether, phenol and amino groups as
described by Gurd, F.R.N. in *Methods Enzymol.* (1967) **11**,
10   532. N-Maleimide derivatives are also considered
selective towards sulfhydryl groups, but may additionaly
be useful in coupling to amino groups under certain
conditions. N-maleimides may be incorporated into
linking systems for reporter-vector conjugation as
15   described by Kitagawa, T. *et al.* in *Chem. Pharm. Bull.*
(1981) **29**, 1130 or used as polymer crosslinkers for
bubble stabilisation as described by Kovacic, P. *et al.*
in *J. Am. Chem. Soc.* (1959) **81**, 1887. Reagents such as
2-iminothiolane, e.g. as described by Traut, R. *et al.*
20   in *Biochemistry* (1973) **12**, 3266, which introduce a thiol
group through conversion of an amino group, may be
considered as sulfhydryl reagents if linking occurs
through the formation of disulphide bridges. Thus
reagents which introduce reactive disulphide bonds into
25   either the reporter or the vector may be useful, since
linking may be brought about by disulphide exchange
between the vector and reporter; examples of such
reagents include Ellman's reagent (DTNB), 4,4'-
dithiodipyridine, methyl-3-nitro-2-pyridyl disulphide
30   and methyl-2-pyridyl disulphide (described by Kimura, T.
et al. in *Analyt. Biochem.* (1982) **122**, 271).

Examples of reactive moieties capable of reaction
with amino groups include alkylating and acylating
agents. Representative alkylating agents include:
35   i)   α-haloacetyl compounds, which show specificity
towards amino groups in the absence of reactive thiol
groups and are of the type X-CH$_2$CO- (where X=Cl, Br or

I), e.g. as described by Wong, Y-H.H. in *Biochemistry* (1979) **24**, 5337;

ii)   N-maleimide derivatives, which may react with amino groups either through a Michael type reaction or through acylation by addition to the ring carbonyl group as described by Smyth, D.G. *et al.* in *J. Am. Chem.* Soc. (1960) **82**, 4600 and *Biochem. J.* (1964) **91**, 589;

iii)   aryl halides such as reactive nitrohaloaromatic compounds;

iv)   alkyl halides as described by McKenzie, J.A. *et al.* in *J. Protein Chem.* (1988) **7**, 581;

v)   aldehydes and ketones capable of Schiff's base formation with amino groups, the adducts formed usually being stabilised through reduction to give a stable amine;

vi)   epoxide derivatives such as epichlorohydrin and bisoxiranes,which may react with amino, sulfhydryl or phenolic hydroxyl groups;

vii)   chlorine-containing derivatives of s-triazines, which are very reactive towards nucleophiles such as amino, sufhydryl and hydroxy groups;

viii) aziridines based on s-triazine compounds detailed above, e.g. as described by Ross, W.C.J. in *Adv. Cancer Res.* (1954) **2**, 1, which react with nucleophiles such as amino groups by ring opening;

ix)   squaric acid diethyl esters as described by Tietze, L.F. in *Chem. Ber.* (1991) **124**, 1215; and

x) α-haloalkyl ethers, which are more reactive alkylating agents than normal alkyl halides because of the activation caused by the ether oxygen atom, e.g. as described by Benneche, T. *et al.* in Eur. J. Med. Chem. (1993) **28**, 463.

Representative amino-reactive acylating agents include:

i)   isocyanates and isothiocyanates, particularly aromatic derivatives, Which form stable urea and thiourea derivatives respectively and have been used for

protein crosslinking as described by Schick, A.F. et al.
in J. Biol. Chem. (1961) 236, 2477;

ii)   sulfonyl chlorides, which have been described by
Herzig, D.J. et al. in Biopolymers (1964) 2, 349 and
which may be useful for the introduction of a
fluorescent reporter group into the linker;

iii)   Acid halides;

iv)   Active esters such as nitrophenylesters or N-
hydroxysuccinimidyl esters;

v)   acid anhydrides such as mixed, symmetrical or N-
carboxyanhydrides;

vi)   other useful reagents for amide bond formation as
described by Bodansky, M. et al. in `Principles of
Peptide Synthesis' (1984) Springer-Verlag;

vii)   acylazides, e.g. wherein the azide group is
generated from a preformed hydrazide derivative using
sodium nitrite, e.g. as described by Wetz, K. et al. in
Anal. Biochem. (1974) 58, 347;

viii)   azlactones attached to polymers such as bis-
acrylamide, e.g. as described by Rasmussen, J.K. in
Reactive Polymers (1991) 16, 199; and

ix)   Imidoesters , which form stable amidines on
reaction with amino groups, e.g. as described by Hunter,
M.J. and Ludwig, M.L. in J. Am. Chem. Soc. (1962) 84,
3491.

Carbonyl groups such as aldehyde functions may be
reacted with weak protein bases at a pH such that
nucleophilic protein side-chain functions are
protonated. Weak bases include 1,2-aminothiols such as
those found in N-terminal cysteine residues, which
selectively form stable 5-membered thiazolidine rings
with aldehyde groups, e.g. as described by Ratner, S. et
al. in J. Am. Chem. Soc. (1937) 59, 200. Other weak
bases such as phenyl hydrazones may be used, e.g. as
described by Heitzman, H. et al. in Proc. Natl. Acad.
Sci. USA (1974) 71, 3537.

Aldehydes and ketones may also be reacted with

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amines to form Schiff's bases, which may advantageously
be stabilised through reductive amination.
Alkoxylamino moieties readily react with ketones and
aldehydes to produce stable alkoxamines, e.g. as
5   described by Webb, R. et al. in Bioconjugate Chem.
(1990) 1, 96.

Examples of reactive moieties capable of reaction
with carboxyl groups include diazo compounds such as
diazoacetate esters and diazoacetamides, which react
10   with high specificity to generate ester groups, e.g. as
described by Herriot R.M. in Adv. Protein Chem. (1947)
3, 169. Carboxylic acid modifying reagents such as
carbodiimides, which react through O-acylurea formation
followed by amide bond formation, may also usefully be
15   employed; linking may be facilitated through addition of
an amine or may result in direct vector-receptor
coupling. Useful water soluble carbodiimides include 1-
cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC)
and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),
20   e.g. as described by Zot, H.G. and Puett, D. in J. Biol.
Chem. (1989) 264, 15552. Other useful carboxylic acid
modifying reagents include isoxazolium derivatives such
as Woodwards reagent K; chloroformates such as p-
nitrophenylchloroformate; carbonyldiimidazoles such as
25   1,1'-carbonyldiimidazole; and N-
carbalkoxydihydroquinolines such as N-(ethoxycarbonyl)-
2-ethoxy-1,2-dihydroquinoline.

Other potentially useful reactive moieties include
vicinal diones such as p-phenylenediglyoxal, which may
30   be used to react with guanidinyl groups, e.g. as
described by Wagner et al. in Nucleic acid Res. (1978)
5, 4065; and diazonium salts, which may undergo
electrophilic substitution reactions, e.g. as described
by Ishizaka, K. and Ishizaka T. in J. Immunol. (1960)
35   85, 163. Bis-diazonium compounds are readily prepared
by treatment of aryl diamines with sodium nitrite in
acidic solutions. It will be appreciated that functional

groups in the reporter and/or vector may if desired be
converted to other functional groups prior to reaction,
e.g. to confer additional reactivity or selectivity.
Examples of methods useful for this purpose include
5   conversion of amines to carboxylic acids using reagents
such as dicarboxylic anhydrides; conversion of amines to
thiols using reagents such as N-acetylhomocysteine
thiolactone, S-acetylmercaptosuccinic anhydride, 2-
iminothiolane or thiol-containing succinimidyl
10   derivatives; conversion of thiols to carboxylic acids
using reagents such as α-haloacetates; conversion of
thiols to amines using reagents such as ethylenimine or
2-bromoethylamine; conversion of carboxylic acids to
amines using reagents such as carbodiimides followed by
15   diamines; and conversion of alcohols to thiols using
reagents such as tosyl chloride followed by
transesterification with thioacetate and hydrolysis to
the thiol with sodium acetate.

Vector-reporter coupling may also be effected
20   using enzymes as zero-length crosslinking agents; thus,
for example, transglutaminase, peroxidase and xanthine
oxidase have been used to produce crosslinked products.
Reverse proteolysis may also be used for crosslinking
through amide bond formation.

25   Non-covalent vector-reporter coupling may, for
example, be effected by electrostatic charge
interactions e.g. between a polylysinyl-functionalised
reporter and a polyglutamyl-functionalised vector,
through chelation in the form of stable metal complexes
30   or through high affinity binding interaction such as
avidin/biotin binding. Polylysine, coated non-
covalently to the negatively charged membrane surface
can also increase non-specifically the affinity of a
microbubble for a cell through charge interactions.

35   Alternatively, a vector may be coupled to a
protein known to bind phospholipids. In many instances,
a single molecule of phospholipid may attach to a

protein such as a translocase, while other proteins may
attach to surfaces consisting mainly of phospholipid
head groups and so may be used to attach vectors to
phospholipid microspheres; one example of such a protein

5   is ß2-glycoprotein I (Chonn, A., Semple, S.C. and
Cullis, P.R., *Journal of Biological Chemistry* (1995)
270, 25845-25849). Phosphatidylserine-binding proteins
have been described, e.g. by Igarashi, K. *et al.* in
Journal of Biological Chemistry **270(49)**, 29075-29078.

10   Annexins are a class of phospholipid-binding proteins,
many of which bind particularly avidly to phosphatidyl-
serine (reviewed in Raynal, P. and H.B. Pollard.
Annexins: the problem of assessing the biological role
for a gene family of multifunctional calcium- and

15   phospholipid-binding proteins". Biochim. Biophys. Acta
1197: 63-93). A conjugate of a vector with such a
phosphatidylserine-binding protein may therefore be used
to attach the vector to phosphatidylserine-encapsulated
microbubbles. When the amino acid sequence of a binding

20   protein is known, the phospholipid-binding portion may
be synthesised or isolated and used for conjugation with
a vector, thus avoiding the biological activity which
may be located elsewhere in the molecule.

It is also possible to obtain molecules that bind

25   specifically to the surface (or in the "membrane") of
microspheres by direct screening of molecular libraries
for microsphere-binding molecules. For example, phage
libraries displaying small peptides could be used for
such selection. The selection may be made by simply

30   mixing the microspheres and the phage display library
and eluting the phages binding to the floating
microspheres. If desired, the selection can be done
under "physiological conditions" (e.g. in blood) to
eliminate peptides which cross-react with blood

35   components. An advantage of this type of selection
procedure is that only binding molecules that do not
destabilize the microspheres should be selected, since

only binding molecules attached to intact floating
microspheres will rise to the top. It may also be
possible to introduce some kind of "stress" during the
selection procedure (e.g. pressure) to ensure that

5   destabilizing binding moieties are not selected.
Furthermore the selection could be done under shear
conditions e.g. by first letting the phages react with
the microspheres and then letting the microspheres pass
through a surface coated with anti-phage antibodies

10   under flow conditions. In this way it may be possible
to select binders which may resist shear conditions
present *in vivo*. Binding moieties identified in this
way may be coupled (by chemical conjugation or via
peptide synthesis, or at the DNA-level for recombinant

15   vectors) to a vector molecule, constituting a general
tool for attaching any vector molecule to the
microspheres.

A vector which comprises or is coupled to a
peptide, lipo-oligosaccharide or lipopeptide linker

20   which contains a element capable of mediating membrane
insertion may also be useful. One example is described
by Leenhouts, J.M. *et al.* in *Febs Letters* (1995) 370(3),
189-192. Non-bioactive molecules consisting of known
membrane insertion anchor/signal groups may also be used

25   as vectors for certain applications, an example being
the H1 hydrophobic segment from the Na,K-ATPase α-
subunit described by Xie, Y. and Morimoto, T. in *J.
Biol. Chem.* (1995) 270(20), 11985-11991. The anchor
group may also be fatty acid(s) or cholesterol.

30   Coupling may also be effected using avidin or
streptavidin, which have four high affinity binding
sites for biotin. Avidin may therefore be used to
conjugate vector to reporter if both vector and reporter
are biotinylated. Examples are described by Bayer, E.A.

35   and Wilchek, M. in *Methods Biochem. Anal.* (1980) 26, 1.

This method may also be extended to include
linking of reporter to reporter, a process which may

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encourage bubble association and consequent potentially
increased echogenicity. Alternatively, avidin or
streptavidin may be attached directly to the surface of
reporter microparticles.

5   Non-covalent coupling may also utilise the
bifunctional nature of bispecific immunoglobulins.
These molecules can specifically bind two antigens, thus
linking them. For example, either bispecific IgG or
chemically engineered bispecific F(ab)'2 fragments may
10   be used as linking agents. Heterobifunctional
bispecific antibodies have also been reported for
linking two different antigens, e.g. as described by
Bode, C. *et al.* in *J. Biol. Chem.* (1989) **264**, 944 and by
Staerz, U.D. *et al.* in *Proc. Natl. Acad. Sci. USA*
15   (1986) **83**, 1453. Similarly, any reporter and/or vector
containing two or more antigenic determinants (e.g. as
described by Chen, Aa *et al.* in *Am. J. Pathol.* (1988)
130, 216) may be crosslinked by antibody molecules and
lead to formation of multi-bubble cross-linked
20   assemblies of potentially increased echogenicity.
Linking agents used in accordance with the
invention will in general bring about linking of vector
to reporter or reporter to reporter with some degree of
specificity, and may also be used to attach one or more
25   therapeutically active agents.
In some instances it is considered advantageous to
include a PEG component as a stabiliser in conjunction
with a vector or vectors or directly to the reporter in
the same molecule where the PEG does not serve as a
30   spacer.
Within the context of the present invention, the
reporter unit will usually remain attached to the
vectors. In another type of targeting procedure,
sometimes called "pre-targeting", the vector (often, a
35   monoclonal antibody) is administered alone;
subsequently, the reporter is administered, coupled to a
moiety which is capable of specifically binding the

vector molecule (when the vector is an antibody, the reporter may be coupled to an immunoglobulin-binding molecule, such as protein A or an anti-immunoglobulin antibody). The advantage of this protocol is that time may be allowed for elimination of the vector molecules that do not bind their targets, substantially reducing the background problems that are connected with the presence of an excess of reporter-vector conjugate. Within the context of the present invention, pre-targeting with one specific vector might be envisaged, followed by reporter units that are coupled to another vector *and* a moiety which binds the first vector.

Within the context of the present invention, in some cases and in particular for the assessment of blood perfusion rates in defined areas, for example in myocardium, it is of interest to measure the rate at which ultrasound contrast agents bound to the target are displaced or released from the target. This can be achieved in a controlled fashion by administration of the vector alone or other agents able to displace or release the ultarsound contrast agent from the target.

Ultrasound imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging and Doppler amplitude imaging, and combinations of the two latter with any of the modalities (techniques) above. Surprisingly, the second harmonic signals from targeted monolayer microspheres were found to be excellent when used in accordance with the present invention. To reduce the

effects of movement, successive images of tissues such
as the heart or kidney may be collected with the aid of
suitable synchronisation techniques (e.g. gating to the
ECG or respiratory movement of the subject).

5   Measurement of changes in resonance frequency or
frequency absorption which accompany arrested or
retarded microbubbles may also usefully be made to
detect the contrast agent

The present invention accordingly provides a tool
10   for therapeutic drug delivery in combination with
vector-mediated direction of the product to the desired
site. By "therapeutic" or "drug" is meant an agent
having a beneficial effect on a specific disease in a
living human or non-human animal. Whilst combinations
15   of drugs and ultrasound contrast agents have been
proposed in, for example, WO-A-9428873 and WO-A-9507072,
these products lack vectors having affinity for
particular sites and thereby show comparitively poor
specific retention at desired sites prior to or during
20   drug release.

Therapeutic compounds used in accordance with the
present invention may be encapsulated in the interior of
the microbubbles/microparticles or attached to or
incorporated into the structure thereof. Thus, the
25   therapeutic compound may be linked to a part of the wall
or matrix, for example through covalent or ionic bonds,
or may be physically mixed into the encapsulating or
matrix material, particularly if the drug has similar
polarity or solubility to this material, so as to
30   prevent it from leaking out of the product before it is
intended to act in the body. The release of the drug
may be initiated merely by wetting contact with blood
following administration or as a consequence of other
internal or external influences, e.g. dissolution
35   processes catalyzed by enzymes or the use of ultrasound.
The destruction of gas-containing microparticles using
external ultrasound is a well known phenomenon in

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respect of ultrasound contrast agents, e.g. as described in WO-A-9325241; the rate of drug release may be varied depending on the type of therapeutic application, using a specific amount of ultrasound energy from the

5   transducer.

The therapeutic may be covalently linked to the membrane or matrix surface using a suitable linking agent, e.g. as described herein. Thus, for example, one may initially prepare a phospholipid or lipopeptide or

10   derivative thereof to which the drug is bonded through a biodegradable bond or linker, and then incorporate this derivative into the material used to prepare the reporter, as described above. Alternatively, the product may initially be prepared without the

15   therapeutic, which may then be coupled to or coated on the microbubbles or microparticles prior to use. Thus, for example, a therapeutic could be added to a suspension of microbubbles or microparticles in aqueous media and shaken in order to attach or adhere the

20   therapeutic thereto.

Exemplary drug delivery systems suitable for use in the present compositions include any known therapeutic drugs or active analogues thereof containing thiol groups which are coupled to thiol containing

25   microbubbles under oxidative conditions yielding disulphide bridges. In combination with a vector or vectors the drug/vector modified microbubbles are allowed to accumulate in the target tissue. Administration of a reducing agent such as reduced

30   glutathione then liberates the drug molecule from the targeted microbubble in the vicinity of the target cell increasing the local concentration of the drug and enhancing therapeutic effect. The product may also be prepared without the therapeutic if desired. The drug

35   may then be coupled to or coated on the microbubbles prior to use. Thus, for example, a therapeutic could be added to a suspension of microbubbles in aqueous media

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and shaken in order to attach or adhere the therapeutic
to the microbubbles.

Other drug delivery systems include vector
modified phospholipid membranes doped with lipopeptide
5   structures comprising a poly-L-lysine or poly-D-lysine
chain in combination with a targeting vector. Applied
to gene therapy/antisense technologies with particular
emphasis on receptor-mediated drug delivery the
microbubble carrier is condensed with DNA or RNA via
10   elecrostatic interaction with the polycation. This
method has the advantage that the vector or vectors used
for targeted delivery are not directly attached to the
polysine carrier moiety. The polylysine chain is also
anchored more tightly in the microbubble membrane due to
15   the presence of the lipid chains. The use of ultrasound
to increase the effectiveness of delivery is also
considered useful.

Alternatively free polylysine chains are firstly
modified with drug or vector molecules then condensed
20   onto the negative surface of targeted microbubbles.

Representative and non-limiting examples of drugs
useful in accordance with the invention include
antineoplastic agents such as vincristine, vinblastine,
vindesine, busulfan, chlorambucil, spiroplatin,
25   cisplatin, carboplatin, methotrexate, adriamycin,
mitomycin, bleomycin, cytosine arabinoside, arabinosyl
adenine, mercaptopurine, mitotane, procarbazine,
dactinomycin (antinomycin D), daunorubicin, doxorubicin
hydrochloride, taxol, plicamycin, aminoglutethimide,
30   estramustine, flutamide, leuprolide, megestrol acetate,
tamoxifen, testolactone, trilostane, amsacrine (m-AMSA),
asparaginase (L-asparaginase), etoposide, interferon a-
2a and 2b, blood products such as hematoporphyrins or
derivatives of the foregoing; biological response
35   modifiers such as muramylpeptides; antifungal agents
such as ketoconazole, nystatin, griseofulvin,
flucytosine, miconazole or amphotericin B; hormones or

hormone analogues such as growth hormone, melanocyte
stimulating hormone, estradiol, beclomethasone
dipropionate, betamethasone, cortisone acetate,
dexamethasone, flunisolide, hydrocortisone,
methylprednisolone, paramethasone acetate, prednisolone,
prednisone, triamcinolone or fludrocortisone acetate;
vitamins such as cyanocobalamin or retinoids; enzymes
such as alkaline phosphatase or manganese superoxide
dismutase; antiallergic agents such as amelexanox;
inhibitors of tissue factor such as monoclonal
antibodies and Fab fragments thereof, synthetic
peptides, nonpeptides and compounds downregulating
tissue factor expression; inhibitors of platelets such
as, GPIa, GPIb and GPIIb-IIIa, ADP receptors, thrombin
receptors, von Willebrand factor, prostaglandins,
aspirin, ticlopidin, clopigogrel and reopro; inhibitors
of coagulation protein targets such as: FIIa FVa, FVIIa,
FVIIIA, FIXa, tissue factor, hepatins, hirudin, hirulog,
argatroban, DEGR-rFVIIa and annexin V; inhibitors of
fibrin formation and promoters of fibrionolysis such as
t-PA, urokinase, Plamin, Streptokinase, rt-Plasminogen
Activator and rStaphylokinase; antiangiogenic factors
such as medroxyprogesteron, pentosan polysulphate,
suramin, taxol, thalidomide, angiostatin, interferon-
alpha, metalloproteinase inhibitors, platelet factor 4,
somatostatin, thromobospondin; circulatory drugs such as
propranolol; metabolic potentiators such as glutathione;
antituberculars such as p-aminosalicylic acid,
isoniazid, capreomycin sulfate, cyclosexine, ethambutol,
ethionamide, pyrazinamide, rifampin or streptomycin
sulphate; antivirals such as acyclovir, amantadine,
azidothymidine, ribavirin or vidarabine; blood vessel
dilating agents such as diltiazem, nifedipine,
verapamil, erythritol tetranitrate, isosorbide
dinitrate, nitroglycerin or pentaerythritol
tetranitrate; antibiotics such as dapsone,
chloramphenicol, neomycin, cefaclor, cefadroxil,

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cephalexin, cephradine, erythromycin, clindamycin,
lincomycin, amoxicillin, ampicillin, bacampicillin,
carbenicillin, dicloxacillin, cyclacillin,
picloxacillin, hetacillin, methicillin, nafcillin,
5   penicillin, polymyxin or tetracycline;
antiinflammatories such as diflunisal, ibuprofen,
indomethacin, meclefenamate, mefenamic acid, naproxen,
phenylbutazone, piroxicam, tolmetin, aspirin or
salicylates; antiprotozoans such as chloroquine,
10   metronidazole, quinine or meglumine antimonate;
antirheumatics such as penicillamine; narcotics such as
paregoric; opiates such as codeine, morphine or opium;
cardiac glycosides such as deslaneside, digitoxin,
digoxin, digitalin or digitalis; neuromuscular blockers
15   such as atracurium mesylate, gallamine triethiodide,
hexafluorenium bromide, metocurine iodide, pancuronium
bromide, succinylcholine chloride, tubocurarine chloride
or vecuronium bromide; sedatives such as amobarbital,
amobarbital sodium, apropbarbital, butabarbital sodium,
20   chloral hydrate, ethchlorvynol, ethinamate, flurazepam
hydrochloride, glutethimide, methotrimeprazine
hydrochloride, methyprylon, midazolam hydrochloride,
paraldehyde, pentobarbital, secobarbital sodium,
talbutal, temazepam or triazolam; local anaesthetics
25   such as bupivacaine, chloroprocaine, etidocaine,
lidocaine, mepivacaine, procaine or tetracaine; general
anaesthetics such as droperidol, etomidate, fentanyl
citrate with droperidol, ketamine hydrochloride,
methohexital sodium or thiopental and pharmaceutically
30   acceptable salts (e.g. acid addition salts such as the
hydrochloride or hydrobromide or base salts such as
sodium, calcium or magnesium salts) or derivatives (e.g.
acetates) thereof. Other examples of therapeutics
include genetic material such as nucleic acids, RNA, and
35   DNA of natural or synthetic origin, including
recombinant RNA and DNA. DNA encoding certain proteins
may be used in the treatment of many different types of

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diseases. For example, tumor necrosis factor or
interleukin-2 genes may be provided to treat advanced
cancers; thymidine kinase genes may be provided to treat
ovarian cancer or brain tumors; interleukin-2 genes may
5   be provided to treat neuroblastoma, malignant melanoma
or kidney cancer; and interleukin-4 genes may be
provided to treat cancer.

Lipophilic derivatives of drugs linked to the
microbubble wall through hydrophobic interactions may
10   exhibit therapeutic effects as part of the microbubble
or after release from the microbubble, e.g. by use of
ultrasound. If the drug does not possess the desired
physical properties, a lipophilic group may be
introduced for anchoring the drug to the membrane.
15   Preferably the lipophilic group should be introduced in
a way that does not influence the *in vivo* potency of the
molecule, or the lipophilic group may be cleaved
releasing the active drug. Lipophilic groups may be
introduced by various chemical means depending on
20   functional groups available in the drug molecule.
Covalent coupling may be effected using functional
groups in the drug molecule capable of reacting with
appropriately functionalised lipophilic compounds.
Examples of lipophilic moieties include branched and
25   unbranched alkyl chains, cyclic compounds, aromatic
residues and fused aromatic and non-aromatic cyclic
systems. In some instances the lipophilic moiety will
consist of a suitably functionalised steroid, like
cholesterol and related compounds. Examples of
30   functional groups particularly suitable for
derivatisation include nucleophilic groups like amino,
hydroxy and sulfhydryl groups. Suitable processes for
lipophilic derivatisation of any drug containing a
sulfhydryl group, like captopril, may include direct
35   alkylation, e.g. reaction with an alkyl halide under
basic conditions and thiol ester formation by reaction
with an activated carboxylic acid. Representative

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examples of derivatisation of any drug having carboxylic
functions, like atenolol and chlorambucil, include amide
and ester formation by coupling of amines and alcohols,
respectively, possesing requested physical properties.

5   A preferred aspect is attachment of cholesterol to a
therapeutic compound by forming a degradable ester bond.

A preferred application of the present invention
relates to *angiogenesis*, which is the formation of new
blood vessels by branching from existing vessels. The

10   primary stimulus for this process may be inadequate
supply of nutrients and oxygen (hypoxia) to cells in a
tissue. The cells may respond by secreting angiogenetic
factors, of which there are many; one example is
vascular endothelial growth factor. These factors

15   initiate the secretion of proteolytic enzymes which
break down the proteins of the basement membrane, as
well as inhibitors which limit the action of these
potentially harmful enzymes. The combined effect of
loss of attachment and signals from the receptors for

20   angiogenetic factors is to cause the endothelial cells
to move, multiply, and rearrange themselves, and finally
to synthetise a basement membrane around the new
vessels.

Tumors must initiate angiogenesis when they reach

25   millimeter size in order to keep up their rate of
growth. As angiogenesis is accompanied by characteristic
changes in the endothelial cells and their environment,
this process is a promising target for therapeutic
intervention. The transformations accompanying

30   angiogenesis are also very promising for diagnosis, a
preferred example being malignant disease, but the
concept also shows great promise in inllammation and a
variety of inflammation-related diseases. These factors
are also involved in re-vascularisation of infarcted

35   parts of the myocardium. which occurs if the stenosis is
released within a short time.

A number of known receptors/targets associated

with angiogenesis are given in subsequent tables. Using the targeting principles described in the present disclosure, angiogenesis may be detected by the majority of the imaging modalities in use in medicine.

5   Contrast-enhanced ultrasound may possess additional advantages, the contrast medium being microspheres which are restricted to the interior of blood vessels. Even if the target antigens are found on many cell types, the microspheres will attach exclusively to endothelial

10   cells.

So-called prodrugs may also be used in agents according to the invention. Thus drugs may be derivatised to alter their physicochemical properties and to adapt them for inclusion into the reporter; such

15   derivatised drugs may be regarded as prodrugs and are usually inactive until cleavage of the derivatising group regenerates the active form of the drug.

By targeting a gas-filled microbubble containing a prodrug-activating enzyme to areas of pathology one may

20   image targeting of the enzyme, making it possible to visualise when the micobubbles are targeted properly to the area of pathology and at the same time have disappeared from non-target areas. In this way one can determine the optimal time for injection of prodrug

25   into individual patients.

Another alternative is to incorporate the prodrug, the prodrug-activating enzyme and the vector in the same microbubble in a system where the prodrug will only be activated after some external stimulus. Such a stimulus

30   may, for example, be a tumour-specific protease as described above, or bursting of the bubbles by external ultrasound after the desired targeting has been achieved.

35   Therapeutics may easily be delivered in accordance with the invention to diseased and necrotic areas including the heart and vasculature in general, and to

the liver, spleen and kidneys and other regions such as
the lymph system, body cavities or gastrointestinal
system.

5   Products according to the present invention may be
     used for targeted therapeutic delivery either *in vivo* or
     in vitro. In the latter context the products may be
     useful in *in vitro* systems such as kits for diagnosis of
     different diseases or characterisation of different
     components in blood or tissue samples. Similar
10   techniques to those used to attach certain blood
     components or cells to polymer particles(e.g.
     monodisperse magnetic particles) *in vitro* to separate
     them from a sample may be used in the present
     invention,using the low density of the reporter units in
15   agents of the present invention to effect separation of
     the gas-containing material by floatation and repeated
     washing.

     So-called zero-length linking agents, which induce
     direct covalent joining of two reactive chemical groups
20   without introducing additional linking material (e.g. as
     in amide bond formation induced using carbodiimides or
     enzymatically) may, if desired, be used in accordance
     with the invention, as may agents such as biotin/avidin
     systems which induce non-covalent reporter-vector
25   linking and agents which induce hydrophobic or
     electrostatic interactions.

     Most commonly, however, the linking agent will
     comprise two or more reactive moieties, e.g. as
     described above, connected by a spacer element. The
30   presence of such a spacer permits bifunctional linkers
     to react with specific functional groups within a
     molecule or between two different molecules, resulting
     in a bond between these two components and introducing
     extrinsic linker-derived material into the reporter-
35   vector conjugate. The reactive moieties in a linking
     agent may be the same (homobifunctional agents) or
     different (heterobifunctional agents or, where several

dissimilar reactive moieties are present,
heteromultifunctional agents), providing a diversity of
potential reagents that may bring about covalent bonding
between any chemical species, either intramolecularly or
5   intermolecularly.

The nature of extrinsic material introduced by the
linking agent may have a critical bearing on the
targeting ability and general stability of the ultimate
product. Thus it may be desirable to introduce labile
10   linkages, e.g. containing spacer arms which are
biodegradable or chemically sensitive or which
incorporate enzymatic cleavage sites. Alternatively the
spacer may include polymeric components, e.g. to act as
surfactants and enhance bubble stability. The spacer
15   may also contain reactive moieties, e.g. as described
above to enhance surface crosslinking, or it may contain
a tracer element such as a fluorescent probe, spin label
or radioactive material.

Contrast agents according to the present invention
20   are therefore useful in all imaging modalities since
contrast elements such as X-ray contrast agents, light
imaging probes, spin labels or radioactive units may
readily be incorporated in or attached to the reporter
units.

25   Spacer elements may typically consist of aliphatic
chains which effectively separate the reactive moieties
of the linker by distances of between 5 and 30 Å. They
may also comprise macromolecular structures such as
poly(ethylene glycols). Such polymeric structures,
30   hereinafter referred to as PEGs, are simple, neutral
polyethers which have been given much attention in
biotechnical and biomedical applications (see e.g.
Milton Harris, J. (ed) "*Poly(ethylene glycol) chemistry,
biotechnical and biomedical applications*" Plenum Press,
35   New York, 1992). PEGs are soluble in most solvents,
including water, and are highly hydrated in aqueous
environments, with two or three water molecules bound to

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each ethylene glycol segment; this has the effect of
preventing adsorption either of other polymers or of
proteins onto PEG-modified surfaces. PEGs are known to
be nontoxic and not to harm active proteins or cells,
whilst covalently linked PEGs are known to be non-
immunogenic and non-antigenic. Furthermore, PEGs may
readily be modified and bound to other molecules with
only little effect on their chemistry. Their
advantageous solubility and biological properties are
apparent from the many possible uses of PEGs and
copolymers thereof, including block copolymers such as
PEG-polyurethanes and PEG-polypropylenes.

Appropriate molecular weights for PEG spacers used
in accordance with the invention may, for example, be
between 120 Daltons and 20 kDaltons.

The major mechanism for uptake of particles by the
cells of the reticuloendothelial system (RES) is
opsonisation by plasma proteins in blood; these mark
foreign particles which are then taken up by the RES.
The biological properties of PEG spacer elements used in
accordance with the invention may serve to increase
contrast agent circulation time in a similar manner to
that observed for PEGylated liposomes (see e.g.
Klibanov, A.L. *et al.* in *FEBS Letters* (1990) **268**, 235-
237 and Blume, G. and Cevc, G. in *Biochim. Biophys. Acta*
(1990) **1029**, 91-97). Increased coupling efficiency to
areas of interest may also be achieved using antibodies
bound to the terminii of PEG spacers (see e.g. Maruyama,
K. *et al.* in *Biochim. Biophys. Acta* (1995) **1234**, 74-80
and Hansen, C.B. *et al.* in *Biochim. Biophys. Acta* (1995)
1239, 133-144).

In some instances it is considered advantageous to
include a PEG component as a stabiliser in conjunction
with a vector or vectors or directly to the reporter in
the same molecule where the PEG does not serve as a
spacer.

Other representative spacer elements include

structural-type polysaccharides such as polygalacturonic
acid, glycosaminoglycans, heparinoids, cellulose and
marine polysaccharides such as alginates, chitosans and
carrageenans; storage-type polysaccharides such as

5   starch, glycogen, dextran and aminodextrans; polyamino
acids and methyl and ethyl esters thereof, as in homo-
and co-polymers of lysine, glutamic acid and aspartic
acid; and polypeptides, oligonucleotides and
oligosaccharides, which may or may not contain enzyme

10   cleavage sites.

In general, spacer elements may contain cleavable
groups such as vicinal glycol, azo, sulfone, ester,
thioester or disulphide groups. Spacers containing
biodegradable methylene diester or diamide groups of

15   formula

$$-(Z)_m.Y.X.C(R^1R^2).X.Y.(Z)_n-$$
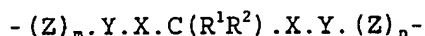
[where X and Z are selected from -O-, -S-, and -NR-
(where R is hydrogen or an organic group); each Y is a
carbonyl, thiocarbonyl, sulphonyl, phosphoryl or similar

20   acid-forming group: m and n are each zero or 1; and R^1
and R^2 are each hydrogen, an organic group or a group
$-X.Y.(Z)_m-$, or together form a divalent organic group]
may also be useful; as discussed in, for example, WO-A-
9217436 such groups are readily biodegraded in the

25   presence of esterases, e.g. in vivo, but are stable in
the absence of such enzymes. They may therefore
advantageously be linked to therapeutic agents to permit
slow release thereof.

Poly[N-(2-hydroxyethyl)methacrylamides] are

30   potentially useful spacer materials by virtue of their
low degree of interaction with cells and tissues (see
e.g. Volfová, I., Ríhová, B. and V.R. and Vetvicka, P.
in J. Bioact. Comp. Polymers (1992) 7, 175-190). Work on
a similar polymer consisting mainly of the closely

35   related 2-hydroxypropyl derivative showed that it was
endocytosed by the mononuclear phagocyte system only to
a rather low extent (see Goddard, P., Williamson, I.,

Bron, J., Hutchkinson, L.E., Nicholls, J. and Petrak, K.
in *J. Bioct. Compat. Polym.* (1991) **6**, 4-24.).

Other potentially useful poymeric spacer materials
include:

5   i)   copolymers of methyl methacrylate with methacrylic
acid; these may be erodible (see Lee, P.I. in *Pharm.
Res.* (1993) **10**, 980) and the carboxylate substituents
may cause a higher degree of swelling than with neutral
polymers;

10   ii)   block copolymers of polymethacrylates with
biodegradable polyesters (see e.g. San Roman, J. and
Guillen-Garcia, P. in *Biomaterials* (1991) **12**, 236-241);

iii)   cyanoacrylates, i.e. polymers of esters of 2-
cyanoacrylic acid - these are biodegradable and have

15   been used in the form of nanoparticles for selective
drug delivery (see Forestier, F., Gerrier, P., Chaumard,
C., Quero, A.M., Couvreur, P. and Labarre, C. in *J.
Antimicrob. Chemoter.* (1992) **30**, 173-179);

iv)   polyvinyl alcohols, which are water-soluble and

20   generally regarded as biocompatible (see e.g. Langer, R.
in *J. Control. Release* (1991) **16**, 53-60);

v)   copolymers of vinyl methyl ether with maleic
anhydride, which have been stated to be bioerodible (see
Finne, U., Hannus, M. and Urtti, A. in *Int. J. Pharm.*

25   *(1992)* **78**. 237-241);

vi)   polyvinylpyrrolidones, e.g. with molecular weight
less than about 25,000, which are rapidly filtered by
the kidneys (see Hespe, W., Meier, A. M. and
Blankwater, Y. M. in *Arzeim.-Forsch./Drug Res.* (1977)

30   **27**, 1158-1162);

vii)   polymers and copolymers of short-chain aliphatic
hydroxyacids such as glycolic, lactic, butyric, valeric
and caproic acids (see e.g. Carli, F. in *Chim. Ind.*
(Milan) (1993) **75**, 494-9), including copolymers which

35   incorporate aromatic hydroxyacids in order to increase
their degradation rate (see Imasaki, K., Yoshida, M.,
Fukuzaki, H., Asano, M., Kumakura, M., Mashimo, T.,

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Yamanaka, H. and Nagai. T. in *Int. J. Pharm.* (1992) **81**, 31-38);

viii) polyesters consisting of alternating units of ethylene glycol and terephthalic acid, e.g. Dacron[R], which are non-degradable but highly biocompatible;

ix) block copolymers comprising biodegradable segments of aliphatic hydroxyacid polymers (see e.g. Younes, H., Nataf, P.R., Cohn, D., Appelbaum, Y.J., Pizov, G. and Uretzky, G. in *Biomater. Artif. Cells Artif. Organs* (1988) **16**, 705-719), for instance in conjunction with polyurethanes (see Kobayashi, H., Hyon, S.H. and Ikada, Y. in "Water-curable and biodegradable prepolymers" - *J. Biomed. Mater. Res.* (1991) **25**, 1481-1494);

x) polyurethanes, which are known to be well-tolerated in implants, and which may be combined with flexible "soft" segments, e.g. comprising poly(tetra methylene glycol), poly(propylene glycol) or poly(ethylene glycol)) and aromatic "hard" segments, e.g. comprising 4,4'-methylenebis(phenylene isocyanate) (see e.g. Ratner, B.D., Johnston, A.B. and Lenk, T.J. in *J. Biomed. Mater. Res: Applied Biomaterials* (1987) **21**, 59-90; Sa Da Costa, V. *et al.* in *J. Coll. Interface Sci.* (1981) **80**, 445-452 and Affrossman, S. *et al.* in *Clinical Materials* (1991) **8**, 25-31);

xi) poly(1,4-dioxan-2-ones), which may be regarded as biodegradable esters in view of their hydrolysable ester linkages (see e.g. Song, C. X., Cui, X. M. and Schindler, A. in *Med. Biol. Eng. Comput.* (1993) **31**, S147-150), and which may include glycolide units to improve their absorbability (see Bezwada, R.S., Shalaby, S.W. and Newman, H.D.J. in *Agricultural and synthetic polymers: Biodegradability and utilization* (1990) (ed Glass, J.E. and Swift, G.), 167-174 - ACS symposium Series, #433, Washington D.C., U.S.A. - American Chemical Society);

xii) polyanhydrides such as copolymers of sebacic acid (octanedioic acid) with bis(4-carboxy-phenoxy)propane,

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which have been shown in rabbit studies (see Brem, H.,
Kader, A., Epstein, J.I., Tamargo, R.J., Domb, A.,
Langer, R. and Leong, K.W. in *Sel. Cancer Ther.* (1989)
5, 55-65) and rat studies (see Tamargo, R.J., Epstein,
J.I., Reinhard, C.S., Chasin, M. and Brem, H. in *J.
Biomed. Mater. Res.* (1989) 23, 253-266) to be useful for
controlled release of drugs in the brain without evident
toxic effects;

xiii) biodegradable polymers containing ortho-ester
groups, which have been employed for controlled release
in vivo (see Maa, Y.F. and Heller, J. in *J. Control.
Release* (1990) 14, 21-28); and

xiv) polyphosphazenes, which are inorganic polymers
consisting of alternate phosphorus and nitrogen atoms
(see Crommen, J.H., Vandorpe, J. and Schacht, E.H. in *J.
Control. Release* (1993) 24, 167-180).

The following tables list linking agents,
biotinylation agents and agents for protein modification
which may be useful in preparing targetable agents in
accordance with the invention.

Heterobifunctional linking agents

Linking agent	Reactivity 1	Reactivity 2	Comments
ABH	carbohydrate	photoreactive	
ANB-NOS	-NH$_2$	photoreactive	
APDP(1)	-SH	photoreactive	iodinable disulphide linker
APG	-NH$_2$	photoreactive	reacts selectively with Arg at pH 7-8
ASIB(1)	-SH	photoreactive	iodinable
ASBA(1)	-COOH	photoreactive	iodinable

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EDC	-NH$_2$	-COOH	zero-length linker
GMBS	-NH$_2$	-SH	
sulfo-GMBS	-NH$_2$	-SH	water-soluble
HSAB	-NH$_2$	photoreactive	
sulfo-HSAB	-NH$_2$	photoreactive	water-soluble
MBS	-NH$_2$	-SH	
sulfo-MBS	-NH$_2$	-SH	water-soluble
M$_2$C$_2$H	carbohydrate	-SH	
MPBH	carbohydrate	-SH	
NHS-ASA(1)	-NH$_2$	photoreactive	iodinable
sulfo-NHS-ASA(1)	-NH$_2$	photoreactive	water-soluble, iodinable
sulfo-NHS-LC-ASA(1)	-NH$_2$	photoreactive	water-soluble, iodinable
PDPH	carbohydrate	-SH	disulphide linker
PNP-DTP	-NH$_2$	photoreactive	
SADP	-NH$_2$	photoreactive	disulphide linker
sulfo-SADP	-NH$_2$	photoreactive	water-soluble disulphide linker
SAED	-NH$_2$	photoreactive	disulphide linker
SAND	-NH$_2$	photoreactive	water-soluble disulphide linker
SANPAH	-NH$_2$	photoreactive	
sulfo-SANPAH	-NH$_2$	photoreactive	water-soluble

SASD(1)	-NH$_2$	photoreactive	water-soluble iodinable disulphide linker
SIAB	-NH$_2$	-SH	
sulfo-SIAB	-NH$_2$	-SH	water-soluble
SMCC	-NH$_2$	-SH	
sulfo-SMCC	-NH$_2$	-SH	water-soluble
SMPB	-NH$_2$	-SH	
sulfo-SMPB	-NH$_2$	-SH	water-soluble
SMPT	-NH$_2$	-SH	
sulfo-LC-SMPT	-NH$_2$	-SH	water-soluble
SPDP	-NH$_2$	-SH	
sulfo-SPDP	-NH$_2$	-SH	water-soluble
sulfo-LC-SPDP	-NH$_2$	-SH	water-soluble
sulfo-SAMCA(2)	-NH$_2$	photoreactive	
sulfo-SAPB	-NH$_2$	photoreactive	water-soluble

Notes: (1)=iodinable; (2)=fluorescent

Homobifunctional linking agents

Linking agent	Reactivity	Comments
BS	-NH$_2$	
BMH	-SH	
BASED(1)	photoreactive	iodinable disulphide linker
BSCOES	-NH$_2$	
sulfo-BSCOES	-NH$_2$	water-soluble
DFDNB	-NH$_2$	
DMA	-NH$_2$	
DMP	-NH$_2$	
DMS	-NH$_2$	

DPDPB	-SH	disulphide linker
DSG	-NH₂	
DSP	-NH₂	disulphide linker
DSS	-NH₂	
DST	-NH₂	
sulfo-DST	-NH₂	water-soluble
DTBP	-NH₂	disulphide linker
DTSSP	-NH₂	disulphide linker
EGS	-NH₂	
sulfo-EGS	-NH₂	water-soluble
SPBP	-NH₂	

Biotinylation agents

Agent	Reactivity	Comments
biotin-BMCC	-SH	
biotin-DPPE*		preparation of biotinylated liposomes
biotin-LC-DPPE*		preparation of biotinylated liposomes
biotin-HPDP	-SH	disulphide linker
biotin-hydrazide	carbohydrate	
biotin-LC-hydrazide	carbohydrate	
iodoacetyl-LC-biotin	-NH₂	
NHS-iminobiotin	-NH₂	reduced affinity for avidin
NHS-SS-biotin	-NH₂	disulphide linker
photoactivatable biotin	nucleic acids	
sulfo-NHS-biotin	-NH₂	water-soluble
sulfo-NHS-LC-biotin	-NH₂	

Notes: DPPE=dipalmitoylphosphatidylethanolamine; LC=long chain

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Agents for protein modification

Agent	Reactivity	Function
Ellman's reagent	-SH	quantifies/detects/protects
DTT	-S.S-	reduction
2-mercaptoethanol	-S.S-	reduction
2-mercaptylamine	-S.S-	reduction
Traut's reagent	-NH$_2$	introduces -SH
SATA	-NH$_2$	introduces protected -SH
AMCA-NHS	-NH$_2$	fluorescent labelling
AMCA-hydrazide	carbohydrate	fluorescent labelling
AMCA-HPDP	-S.S-	fluorescent labelling
SBF-chloride	-S.S-	fluorescent detection of -SH
N-ethylmaleimide	-S.S-	blocks -SH
NHS-acetate	-NH$_2$	blocks and acetylates -NH$_2$
citraconic anhydride	-NH$_2$	reversibly blocks and introduces negative charges
DTPA	-NH$_2$	introduces chelator
BNPS-skatole	tryptophan	cleaves tryptophan residue
Bolton-Hunter	-NH$_2$	introduces iodinable group

Other potentially useful protein modifications
include partial or complete deglycosidation by
neuraminidase, endoglycosydases or periodate, since
deglycosidation often results in less uptake by liver,
spleen, macrophages etc., whereas neo-glycosylation of
proteins often results in increased uptake by the liver
and macrophages); preparation of truncated forms by
proteolytic cleavage, leading to reduced size and
shorter half life in circulation; and cationisation,
e.g. as described by Kumagi et al. in J. Biol. Chem.
(1987) 262, 15214-15219; Triguero et al. in Proc. Natl.
Acad. Sci. USA (1989) 86, 4761-4765; Pardridge et al. in
J. Pharmacol. Exp. Therap. (1989) 251, 821-826 and

Pardridge and Boado, *Febs Lett.* (1991) **288**, 30-32.

Vectors which may be usefully employed in
targetable agents according to the invention include the
following:

5

i)   Antibodies, which can be used as vectors for a
very wide range of targets, and which have advantageous
properties such as very high specificity, high affinity
(if desired), the possiblity of modifying affinity
10   according to need etc. Whether or not antibodies will
be bioactive will depend on the specific vector/target
combination. Both conventional and genetically
engineered antibodies may be employed, the latter
permitting engineering of antibodies to particular
15   needs, e.g. as regards affinity and specificity. The
use of human antibodies may be preferred to avoid
possible immune reactions against the vector molecule.
A further useful class of antibodies comprises so-called
bi- and multi-specific antibodies, i.e. antibodies
20   having specificity for two Or more different antigens in
one antibody molecule. Such antibodies may, for
example, be useful in promoting formation of bubble
clusters and may also be used for various therapeutic
purposes, e.g. for carrying toxic moieties to the
25   target. Various aspects of bispecific antibodies are
described by McGuinness, B.T. *et al.* in *Nat. Biotechnol.*
(1996) **14**, 1149-1154; by George, A.J. *et al.* in *J.
Immunol.* (1994) **152**, 1802-1811; by Bonardi *et al.* in
Cancer Res. (1993) **53**, 3015-3021; and by French, R.R. *et
30   al.* in *Cancer Res.* (1991) **51**, 2353-2361.

ii)   Cell adhesion molecules, their receptors,
cytokines, growth factors, peptide hormones and
fragments and pieces thereof. Such vectors rely on
35   normal biological protein-protein interactions with
their target molecules, and so in many cases will
generate a biological response on binding with the

targets and thus be bioactive; this may be a relatively insignificant concern with vectors which target proteoglycans.

5   iii) Non-bioactive binders of receptors for cell adhesion molecules, cytokines, growth factors or peptide hormones. This category may include peptidic or non-peptidic non-bioactive vectors which will be neither agonists nor antagonists.

10

iv) Oligonucleotides and modified oligonucleotides which bind DNA or RNA through Watson-Crick or other types of base-pairing. DNA is usually only present in extracelluar space as a consequence of cell damage, so
15   that such oligonucleotides, which will usually be non-bioactive, may be useful in, for example, targeting of necrotic regions, which are associated with many different pathological conditions. Oligonucleotides may also be designed to bind to specific DNA- or RNA-binding
20   proteins, for example transcription factors which are very often highly overexpressed or activated in tumour cells or in activated immune or endothelial cells. Combinatorial libraries may be used to select oligonucleotides which bind specifically to any possible
25   target molecules (from the examples of proteins to caffeine) and which therefore may be employed as vectors for targeting.

v) DNA-binding drugs may behave similarly to
30   oligonuclotides, but may exhibit biological acitvity and/or toxic effects if taken up by cells.

vi) Protease substrates/inhibitors. Proteases are involved in many pathological conditions. Many
35   substrates/inhibitors are non-peptidic but, at least in the case of inhibitors, are often bioactive.

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vii)   Vector molecules may be generated from
combinatorial libraries without necessarily knowing the
exact molecular target, by functionally selecting (in
vitro, ex vivo or in vivo) for molecules binding to the
5   region/structure to be imaged.

viii) Various small molecules, including bioactive
compounds known to bind to biological receptors of
various kinds. Such vectors or their targets may be
10   used for generate non-bioactive compounds binding to the
same targets.

ix) proteins or peptides which bind to glucosaminoglycan
side chains eg heparan sulphate, including
15   glucosoaminoglycan-binding portions of larger molecules,
as binding to glucosoaminoglycans does not result in a
biological response. Proteoglycans are not found on red
blood cells, which eliminates undesirable adsorption to
these cells.
20   The following tables identify various vectors
which may be targeted to particular types of targets and
indicated areas of use for targetable diagnostic and/or
therapeutic agents according to the invention which
contain such vectors. In the cases where the vectors
25   stated are bioactive it is understood that either: (1)
non-bioactive analogs are used; (2) the vectors are used
in doses too low to generate a biological response; or
(3) the vectors are used in combinations in such a way
that the resulting diagnostic and/or therapeutic
30   composition gives no biological response.

Protein and peptide vectors - antibodies

Vector type	Target	Comments/areas of use	Ref

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antibodies (general)	CD34	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells	1
"	ICAM-1	"	1
"	ICAM-2	"	1
"	ICAM-3	"	1
"	E-selectin	"	1
"	P-selectin	"	1
"	PECAM	"	1
"	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	"	2
"	GlyCAM	Vessel wall in lymph nodes (quite specific for lymph nodes)	3
"	MadCam 1	"	3
"	fibrin	Thrombi	4
"	Tissue Factor	Activated endothelium, tumours	5
"	Myosin	Necrosis, myocardial infaction	6
"	CEA (carcino-embryonal antigen)	Tumours	7
"	Mucins	Tumours	8
"	Multiple drug resistance protein	Tumours	9
"	Prostate specific antigen	Prostate cancer	

	Cathepsin B	Tumours (proteases of various kinds are often more or less specifically overexpressed in a variety of tumours - Cathepsin B is such a protease)	10
"	Transferrin receptor	Tumors, vessel wall	11
MoAb 9.2.27		Tumours Antigen upregulated on cell growth	12
	VAP-1	Adhesion molecule	13
	Band 3 protein	Upregulated during phagocytic activity	
antibodies	CD44	tumour cells	a
antibodies	β2-micro-globulin	general	b
antibodies	MHC class 1	general	b
antibodies	integrin αvβ3	tumours, angiogenesis	c

a.) Heider, K. H., M. Sproll, S. Susani, E. Patzelt, P. Beaumier, E. Ostermann, H. Ahorn, and G. R. Adolf. **1996.** "Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas". *Cancer Immunology Immunotherapy* **43**: 245-253.

b). I. Roitt, J. Brostoff, and D. Male. **1985.** *Immunology*, London: Gower Medical Publishing, p. 4.7

c.) Stromblad, S., and D. A. Cheresh. **1996.** "Integrins, angiogenesis and vascular cell survival". *Chemistry & Biology* **3**: 881-885.

Protein and peptide vectors - cell adhesion molecules etc.

Vector type	Target	Comments/areas of use	Ref

L-selectin	CD34 MadCAM1 GlyCam 1	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, Lymph nodes	3
Other selectins	carbohydrate ligands (sialyl Lewis x) heparan sulfate	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium	14
RGD-peptides	integrins	"	2
PECAM	PECAM, and other	Endothelium, Cells in immune system	15
Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Laminin, collagen, fibronectin, VCAM-1, thrombo-spondin, vitronectin etc.	Endothelium, Vessel wall etc.	16
Integrin receptors, e.g.Laminin, collagen, fibronectin, VCAM-1, thrombospondin, vitronectin etc.	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Cells in immune system vessel wall etc.	1718
Nerve cell adhesion molecule (N-CAM) RGD-peptides	proteoglycans N-CAM (homophilic) integrins	angiogenesis	19 c

Vectors comprising cytokines/growth factors/peptide hormones and fragments thereof

	Vector type	Target	Comments/areas of use	Ref
	Epidermal growth factor	EGF-receptor or related receptors	Tumours	20
5	Nerve growth factor	NGF-receptor	Tumours	21
	Somatostatin	ST-receptor	Tumours	22
	Endothelin	Endothelin-receptor	Vessel wall	
	Interleukin-1	IL-1-receptor	Inflammation, activated cells of different kinds	23
	Interleukin-2	IL-2-receptor	"	24
10	Chemokines (ca. 20 different cytokines partly sharing receptors)	Chemokine receptors, proteoglycans	Inflammation	25
15	Tumour necrosis factor	TNF-receptors	Inflammation	
	Parathyroid hormone	PTH-receptors	Bone diseases Kidney diseases	
20	Bone Morphogenetic Protein	BMP-receptors	Bone Diseases	
	Calcitonin	CT-receptors	Bone diseases	
25	Colony stimulating factors (G-CSF, GM-CSF, M-CSF, IL-3)	Corresponding specific receptors, proteoglycans	Endothelium	26
	Insulin like growth factor I	IGF-I receptor	Tumours, other growing tissues	
30	Atrial Natriuretic Factor	ANF-receptors	Kidney, vessel wall	

Vasopressin	Vasopressin receptor	Kidney, vessel wall	
VEGF	VEGF-receptor	Endothelium, regions of angiogenesis	
Fibroblast growth factors	FGF-receptors, Proteoglycans	Endothelium Angiogenesis	27
Schwann cell growth factor	proteoglycans specific receptors		28

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Miscellaneous protein and peptide vectors

Vector type	Target	Comments/areas of use	Ref
Streptavidin	Kidney	Kidney diseases	29
Bacterial fibronectin-binding proteins	Fibronectin	Vessel wall	30
Fc-part of antibodies	Fc-receptors	Monocytes macrophages liver	31
Transferrin	transferrin-receptor	Tumours vessel walls	11
Streptokinase/ tissue plasminogen activator	thrombi	thrombi	
Plasminogen, plasmin	Fibrin	Thrombi, tumours	32
Mast cell proteinases	proteoglycans		33
Elastase	proteoglycans		34
Lipoprotein lipase	proteoglycans		35
Coagulation enzymes	proteoglycans		36
Extracellular superoxide dismutase	proteoglycans		37
Heparin cofactor II	proteoglycans		38
Retinal survival factor	proteoglycans specific receptors		39

Heparin-binding brain mitogen	proteoglycans specific receptors		40
Apolipoprotein, e.g. apolipoprotein B	proteoglycans specific receptors (e.g., LDL receptor)		41
Apolipoprotein E	LDL receptor proteoglycans		42
Adhesion-promoting proteins, e.g. Purpurin	proteoglycans		43
Viral coat proteins, e.g. HIV, Herpes	proteoglycans		44
Microbial adhesins, e.g. "Antigen 85" complex of mycobacteria	fibronectin, collagen, fibrinogen, vitronectin, heparan sulfate		45
ß-amyloid precursor	proteoglycans	ß-amyloid accumulates in Alzheimer's disease	46
Tenascin, e.g .tenascin C	heparan sulfate, integrins		47

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Vectors comprising non-peptide agonists/antagonists or non-bioactive binders of receptors for cytokines/growth factors/peptide hormones/cell adhesion molecules

Vector type	Target	Comments/areas of use	Ref
		Several agonists/antagonists are known for such factors acting through G-protein coupled receptors	48 49
Endothelin antagonist	Endothelin receptor	Vessel wall	
Desmopressin (vasopressin analogue)	Vasopressin receptor	Kidney Vessel wall	
Demoxytocin (oxytocin analogue)	Oxytocin Receptor	Reproductive organs, Mammary glands, Brain	
Angiotensin II receptor antagonists CV-11974, TCV-116	Angiotensin II receptors	Vessel wall brain adrenal gland	
non-peptide RGD-analogues	integrins	Cells in immune system vessel wall etc.	50

Vectors comprising anti-angiogenic factors

Vector type	Target	Comments/areas of use	Ref
Angiostatin	EC of tumors	plasminogen fragment	K
cartilage-derived inhibitor	EC of tumors		J

β-Cyclodextrin tetradecasulfate	tumors, inflammation		C
fumagillin and analogs	tumors, inflammation		E
Interferon-α	EC of tumors		K
Interferon-γ	EC of tumors		E
interleukin-12	EC of tumors		E
linomide	tumors, inflammation		A
medroxyprogesterone	EC of tumors		K
metalloproteinase inhibitors	EC of tumors		K
pentosan polysulfate	EC of tumors		K
platelet factor 4	EC of tumors		M
Somatostatin	EC of tumors		K
Suramin	EC of tumors		K
Taxol	EC of tumors		K
thalidomide	EC of tumors		K
Thrombospondin	EC of tumors		K

Vectors comprising angiogenic factors

Vector type	Target	Comments/areas of use	Ref
acidic fibroblast growth factor	EC of tumors		K
adenosine	EC of tumors		K
Angiogenin	EC of tumors		K
Angiotensin II	EC of tumors		K
basement membrane components	tumors	e.g., tenascin, collagen IV	M
basic fibroblast growth factor	EC of tumors		K

Bradykinin	EC of tumors		K
Calcitonin gene-related peptide	EC of tumors		K
epidermal growth factor	EC of tumors		K
Fibrin	tumors		K
Fibrinogen	tumors		K
Heparin	EC of tumors		K
histamine	EC of tumors		K
hyaluronic acid or fragments thereof	EC of tumors		K
Interleukin-1α	EC of tumors		K
laminin, laminin fragments	EC of tumors		K
nicotinamide	EC of tumors		K
platelet activating factor	EC of tumors		K
Platelet-derived endothelial growth factor	EC of tumors		K
prostaglandins E1, E2	EC of tumors		K
spermine	EC of tumors		K
spermine	EC of tumors		K
Substance P	EC of tumors		K
transforming growth factor-α	EC of tumors		K
transforming growth factor-β	EC of tumors		K
Tumor necrosis factor-α	EC of tumors		K
vascular endothelial growth factor/vascular permeability factor	EC of tumors		K
vitronectin			A

Vector molecules other than recognized angiogenetic factors with known affinity for receptors associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
angiopoietin	tumors, inflammation		B
α_2-antiplasmin	tumors, inflammation		
combinatorial libraries, compounds from	tumors, inflammation	for instance: compounds that bind to basement membrane after degradation	
endoglin	tumors, inflammation		D
endosialin	tumors, inflammation		D
endostatin [collagen fragment]	tumors, inflammation		M
Factor VII related antigen	tumors, inflammation		D
fibrinopeptides	tumors, inflammation		ZC
fibroblast growth factor, basic	tumors, inflammation		E
hepatocyte growth factor	tumors, inflammation		I
insulin-like growth factor	tumors, inflammation		R
interleukins	tumors, inflammation	e.g.,: IL-8	I
leukemia inhibitory factor	tumors, inflammation		A

	metalloproteinase inhibitors	tumors, inflammation	e.g., batimastat	E
	Monoclonal antibodies	tumors, inflammation	for instance: to angiogenetic factors or their receptors, or to components of the fibrinolytic system	
5	peptides, for instance cyclic RGDᴅFV	tumors, inflammation		B,Q
	placental growth factor	tumors, inflammation		J
	placental proliferin-related protein	tumors, inflammation		E
10	plasminogen	tumors, inflammation		M
	plasminogen activators	tumors, inflammation		D
	plasminogen activator inhibitors	tumors, inflammation		U,V
15	platelet activating factor antagonists	tumors, inflammation	inhibitors of angiogenesis	A
	platelet-derived growth factor	tumors, inflammation		E
	pleiotropin	tumors, inflammation		ZA
	proliferin	tumors, inflammation		E
20	proliferin related protein	tumors, inflammation		E
	selectins	tumors, inflammation	e.g., E-selectin	D
	SPARC	tumors, inflammation		M

snake venoms (RGD-containing)	tumors, inflammation		Q
Tissue inhibitor of metalloproteinases	tumors, inflammation	e g,, TIMP-2	U
thrombin	tumors, inflammation		H
thrombin-receptor-activati ng tetradecapeptide	tumors, inflammation		H
thymidine phosphorylase	tumors, inflammation		D
tumor growth factor	tumors, inflammation		ZA

Receptors/targets associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
biglycan	tumors, inflammation	dermatan sulfate proteoglycan	X
CD34	tumors, inflammation		L
CD44	tumors, inflammation		F
collagen type I, IV, VI, VIII	tumors, inflammation		A
decorin	tumors, inflammation	dermatan sulfate proteoglycan	Y
dermatan sulfate proteoglycans	tumors, inflammation		X
endothelin	tumors, inflammation		G
endothelin receptors	tumors, inflammation		G

fibronectin	tumors	.	P
Flk-1/KDR, Flt-4	tumors, inflammation	VEGF receptor	D
FLT-1 (fms-like tyrosine kinase)	tumors, inflammation	VEGF-A receptor	O
heparan sulfate	tumors, inflammation		p
hepatocyte growth factor receptor (c-met)	tumors, inflammation		I
insulin-like growth factor/mannose-6-phosphate receptor	tumors, inflammation		R
integrins: β_3 and β_5, integrin $\alpha_v\beta_3$, integrin $\alpha_6\beta_1$, , integrins α_6, integrins β_1, integrin $\alpha_2\beta_1$, integrin $\alpha_v\beta_3$, integrin α_5 integrin $\alpha_v\beta_5$, fibrin receptors.	Tumors, inflammation	laminin receptor subunit of the fibronectin receptor	D, P
Intercellular adhesion molecule-1 and -2	tumors, inflammation		P
Jagged gene product	tumors, inflammation		T
Ly-6	tumors, inflammation	a lymphocyte activation protein	N
matrix metalloproteinases	tumors, inflammation		D
MHC class II	tumors, inflammation		

Notch gene product	tumors, inflammation		T
Osteopontin	tumors		Z
PECAM	tumors, inflammation	alias CD31	P
plasminogen activator receptor	tumors, inflammation		ZC
platelet-derived growth factor receptors	tumors, inflammation		E
Selectins: E-, P-	tumors, inflammation		D
Sialyl Lewis-X	tumors, inflammation	blood group antigen	M
stress proteins: glucose regulated, heat shock families and others	tumors, inflammation	molecular chaperones	
syndecan	tumors, inflammation		T
thrombospondin	tumors, inflammation		M
TIE receptors	tumors, inflammation	tyrosine kinases with Ig- and EGF-Iike domains	E
tissue factor	tumors, inflammation		Z
tissue inhibitor of metalloproteinases	tumors, inflammation	e.g., TIMP-2	U
transforming growth factor receptor	tumors, inflammation		E
urokinase-type plasminogen activator receptor	tumors, inflammation		D
Vascular cellular adhesion molecule (VCAM)	tumors, inflammation		D

Vascular endothelial growth factor related protein	tumors, inflammation		
Vascular endothelial growth factor-A receptor	tumors, inflammation		K
von Willebrand factor-related antigen	tumors, inflammation		L

Oligonucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Oligonucleotides complementary to repeated sequences, e.g. genes for ribosomal RNA, Alu-sequences	DNA made available by necrosis	Tumours Myocardial infarction All other diseases that involves necrosis	51
Oligonucleotides complementary to disease-specific mutations (e.g. mutated oncogenes).	DNA made available by necrosis in a region of the relevant disease	Tumours	51
Oligonucleotides complementary to DNA of infecting agent.	DNA of infective agent	Viral or bacterial infections	51
Triple or quadruple-helix forming oligonucleotides	As in above examples	As in above examples	51

Oligonucleotides with recognition sequence for DNA-or RNA- binding proteins	DNA-binding protein, e.g. transcription factors (often overexpressed/ activated in tumours or activated endothelium/ immune cells	Tumours Activated endothelium Activated immune cells	

Modified oligonucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Phosphorothioate oligos	As for unmodified oligos	As for unmodified oligos	51
2'-O-methyl substituted oligos	"	"	51
circular oligos	"	"	51
oligos containing hairpin structure to decrease degradation	"	"	51
oligos with terminal phosphorothioate	"	"	51
2'-fluoro oligos	"	"	51
2'-amino oligos	"	"	51
DNA-binding drugs conjugated to oligos (for examples, see below)	"	Increased binding affinity as compared to pure oligos	52

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Peptide Nucleic Acids (PNAs, oligonucleotidss with a peptide backbone)	"	Increased binding affinity and stability compared to standard oligos.	53

5

Nucleoside and nucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Adenosine or analogues	Adenosine receptors	Vessel wall Heart	54
ADP, UDP, UTP and others	Various nucleotide receptors	Many tissues, e.g. brain, spinal cord, kidney, spleen	55

10 (row label for ADP row)

Receptors comprising DNA-binding drugs

15

Vector type	Target	Comments/areas of use	Ref
acridine derivatives distamycin netropsin actinomycin D echinomycin bleomycin etc.	DNA made available by necrosis	Tumours, Myocardial infarction and all other diseases involving necrosis or other processes liberating DNA from cells	

20

Receptors comprising protease substrates

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic substrates	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10

Receptors comprising protease inhibitors

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic inhibitors e.g. N-acetyl-Leu-Leu-norleucinal	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10
bestatin ([(2S,3R)-3-Amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine hydrochloride)	Aminopeptidases	Tumours, e.g. on cell surfaces	
Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)	Serine proteases	Tumours, vessel wall etc.	
Commercially available inhibitors e.g. kaptopril enalapril ricionopril	Angiotensin converting enzyme	Endothelial cells	

Low specificity non-peptidic compounds	Coagulation factors	Vessel wall injury, tumours, etc.	
Protease nexins (extracellular protease inhibitors)	proteoglycans		56
Antithrombin	proteoglycans, Coagulation factors	.	57

Vectors from combinatorial libraries

Vector type	Target	Comments/areas of use	Ref
Antibodies with structure determined during generation process	Any of above targets - or may be unknown when make functional selection of vector binding to chosen diseased structure	Any diseased or normal structure of interest, e.g. thrombi, tumours or walls of myocardial vessels	58, 59, 60
Peptides with sequence determined during generation process	"	"	58, 59, 60
Oligonucleotides with sequence determined during generation process	"	"	58, 59, 60
Modifications of oligos obtained as above	"	"	58, 59, 60

Other chemicals with structure determined during generation process	"	"	58, 59, 60

5

Carbohydrate vectors

Vector type	Target	Comments/areas of use	Ref
neo-glycoproteins	macrophages	general activation/ inflammation	
oligosaccharides with terminal galactose	Asialo-glycoprotein receptor	liver	61
Hyaluronan	aggrecan (a proteoglycan) "link proteins" cell-surface receptors: CD44		62
Mannose		Blood brain barrier, Brain tumours and other diseases causing changes in BBB	63
Bacterial glycopeptides		"	64

20

(Glyco)Lipid vectors

Vector type	Target	Comments/areas of use	Ref
GM1 gangliosides	cholera bacteria in the gastrointestinal tract	diagnosis/treatment of cholera	

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platelet activating factor (PAF) antagonists	PAF receptors	diagnosis of inflammation	
Prostoglandin antagonists of inflammation	Prostoglandin receptors	diagnosis of inflammation	
Thromboxane antagonists of inflammation	Leukotriene receptors	diagnosis of inflammation	

Small molecule vectors

Vector type	Target	Comments/areas of use	Ref
Adrenalin	Corresponding receptors		
Betablockers	Adrenergic beta-receptors	Myocardium for beta-1 blockers	
Alpha-blockers	Adrenergic alpha-receptors	Vessel wall	
benzodiazepines			
serotonin-analogues	Serotonin-receptors		
anti-histamines	Histamine-receptors	Vessel wall	
Acetyl-choline receptor antagonists	ACh-receptors		
verapamil	Ca^{2+}-channel blocker	Heart muscle	
nifedipin	Ca^{2+}-channel blocker	Heart muscle	

Amiloride	Na$^+$/H$^+$-exchanger	Blocks this exchanges in kidney and is generally upregulated in cells stimulated by growth factors.	
Digitalis glycosides	Na$^+$/K$^+$-ATP-ases	myocardium peripheral vasculature, central nervous system	
Thromboxae/ Prostaglandin receptor antagonists or agonists	Thromboxane/ prostaglandin receptors	Vessel wall, Endothelium	
Glutathione	Glutathione-receptors Leukotriene-receptors	Lung, Brain	
Biotin	biotin transport protein on cell surface		65
Folate	folate transport protein on cell surface	Tumours	66
Riboflavin	riboflavin transport protein on cell surface		67

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References

1.   Nourshargh, S. and Williams, T.J. (1995). Semin. Cell Biol. *6*, 317-326.

2.   Simmons, D.L. (1996). TIBTECH *14*, 221-222.

3.   Baumhueter, S., Dybdal, N., Kyle, C., and Lasky, L.A. (1994). Blood *84*, 2554-2565.

4.   Rosenthall, L. and Leclerc, J. (1995).Clin. Nucl. Med. *20*, 398-402.

5.   Contrino, J., Hair, G., Kreutzer, D.L., and Rickles, F.R. (1996). Nature Med *2*, 209-215.

6.   Torchilin, V.P., Narula, J., Halpern, E., and Khaw, B.A. (1996). Biochim. Biophys. Acta *1279*, 75-83.

7.   Wittig, B.M., Hach, A., Hahn, K., Meyer zum, B., KH, and Dippold, W.G. (1993). Eur. J. Cancer *29A*, 1327-1329.

8.   Mariani, G., Molea, N., Bacciardi, D., Boggi, U., Fornaciari, G., Campani, D., Salvadori, P.A., Giulianotti, P.C., Mosca, F., and Gold, D.V. (1995). Cancer Res. *55*, 5911s-5915s.

9.   Scott, A.M., Rosa, E., Mehta, B.M., Divgi, C.R., Finn, R.D., Biedler, J.L., Tsuruo, T., Kalaigian, H., and Larson, S.M. (1995). Nucl. Med. Biol. *22*, 497-504.

10.   Panchal, R.G. et al. (1996), Nat. Biotechnol. **14**, 852-856.

11.   Wagner, E. et al. (1994), Adv. Drug Deliv. Rev. **14**, 113-115.

12.   Ballou, B., Fisher, G.W., Waggoner, A.S., Farkas, D.L., Reiland, J.M., Jaffe, R., Mujumdar, R.B., Mujumdar, S.R., and Hakala, T.R. (1995). Cancer Immunol. Immunother. *41*, 257-263.

13.   Salmi, M. and Jalkanen, S. (1995). Eur. J. Immunol. *25*, 2803-2812.

14.   McEver, R.P. **1992.** *Curr. Opin. Cell Biol.* **4,** 840-49.

15.   DeLisser, H.M., Newman, P.J., and Albelda, S.A. (1994). Immunol. Today *15*, 490-495.

16.   Metcalf, B.W. et al., eds. (1994): Cellular Adhesion. Molecular Definition to Therapeutic Potential. Plenum Press, New York, 1994.

17.   Felding-Habermann, B. and Cheresh, D.A. **1993.** *Curr. Opin. Cell Biol.* **5,** 864-868.

18.   Schwarzbauer, J.E. **1991.** *Curr. Opin. Cell Biol.* **3,** 786-791.

19.   Burg, M.A., Halfter, W., Cole, G.J. **1995.** *J. Neurosci. Res.* **41,** 49-64.

20.   Dean, C.J., Eccles, S.A., Valeri, M., Box, G., Allan, S., McFarlane, C., Sandle, J., and Styles, J. (1993).Cell Biophys. *22*, 111-127.

21.   LeSauteur, L. et al. (1996), Nat. Biotechnol. **14,** 1120-1122.

22.   Wiseman, G.A. and Kvols, L.K. (1995). Semin. Nucl. Med. *25*, 272-278.

23.   van der Laken, C.J., Boerman, O.C., Oyen, W.J., van
de Ven, M.T., Claessens, R.A., van der Meer, J.W., and
Corstens, F.H. (1995). Eur. J. Nucl. Med. *22*, 1249-1255.

24.   Signore, A., Chianelli, M., Ferretti, E., Toscano,
A., Britton, K.E., Andreani, D., Gale, E.A., and
Pozzilli, P. (1994). Eur. J. Endocrinol. *131*,

25.   Howard, O.M.Z. et al. (1996), TIBTECH **14**, 46-51.

26.   Korpelainen, E.I., Gamble, J.R., Smith, W.B.,
Dottore, M., Vadas, M.A., and Lopez, A.F. (1995). Blood
86, 176-182.

27.   Klagsbrun, M. **1990.** "*Curr. Opin. Cell Biol.* **2:**
857-863.

28.   Ratner, N., D. Hong, M. A. Lieberman, R. P. Bunge,
and L. Glaser. **1988.** *Proc. Natl. Acad. Sci. USA* **85:**
6992-6.

29.   Schechter, B., Arnon, R., Colas, C., Burakova, T.,
and Wilchek, M. (1995). KIDNEY INTERNATIONAL. 47,
1327-35.

30.   Valentin-Weigand, P., Talay, S., R, Kaufhold, A.,
Timmis, K., N, and Chhatwal, G., S (1994). MICROBIAL.
PATHOGENESIS. *17*, 111-20.

31.   Betageri, G.V., Black, C.D., Szebeni, J., Wahl,
L.M., and Weinstein, J.N. (1993). J. Pharm. Pharmacol.
45, 48-53.

32.   Blume, G., Cevc, G., Crommelin, M.D.,
Bakker-Woudenberg, I.A., Kluft, C., and Storm, G.
(1993). Biochim. Biophys. Acta *1149*, 180-184.

- 71 -

33.   Stevens, R. L., and K. F. Austen. **1989.** *Immunology Today* **10**: 381-386

34.   Redini, F., J. M. Tixier, M. Petitou, J. Chouay, L. Robert, and W. Hornebeck. **1988.** *Biochem. J.* **252**: 515-19.

35.   Persson, B., O. G. Bengtsson, S. Enerbäck, T. Olivecrona, and H. Jörnvall. **1989.** *Eur. J. Biochem.* **179**: 39-45.

36.   Danielsson, Å., E. Raub, U. Lindahl, and I. Bj¢rk. **1986.** *J. Biol. Chem.* **261**: 15467-73.

37.   Adachi, T., and S. L. Marklund. **1989.** *J. Biol. Chem.* **264**: 8537-41.

38.   Maimone, M. M., and D. M. Tollefsen. **1990.** *J. Biol. Chem.* **265**: 18263-71.

39.   Berman, P., P. Gray, E. Chen, K. Keyser, and D. e. a. Eherlich. **1987.** *Cell* **51**: 135-42.

40.   Huber, D., P. Gautschi-Sova, and P. Böhlen. **1990.** *Neurochem. Res.* **15**: 435-439

41.   P. Vijayagopal, B. Radhnakrishnamurthy, G.S. Berenson. **1995.** *Biochim. Biophys. Acta* **1272**: 61-67.

42.   Dyer, C. A., and L. K. Curtiss. **1991..** *J. Biol. Chem.* **266** (34): 22803-22806.

43.   Rauvala, H., J. Merenmies, R. Pihlaskari, M. Kormalainen, M. L. Huhtala, and P. Panula. **1988.** *J. Cell Biol.* **107**: 2293-2305.

44.   WuDunn, D., and P. G. Spear. **1989.** *J. Virol.* **63**: 52-58.

- 72 -

45.   Patti, J. M., and M. Höök. **1994.** *Curr. Opin. Cell Biol.* **6**: 752-758.

46.   Snow, A.D., M.G. Kinsella, E. Parks, R.T. Sekiguchi et al. **1995.** *Arch. Biochem. Biophys.* **320**: 84-95

47.   Fischer, D., R. Chiquet-Ehrismann, C. Bernasconi, M. Chiquet. **1995.** *J. Biol. Chem.* **270**: 3378-84.

48.   Wells, J.A. (1996), Science **273**, 449-450.

49.   Longo, F.M. and Mobley, W.C (1996), Nat. Biotechnol. **14**, 1092.

50.   Samanen, J.M. et al. (1994), in Metcalf, B.W. et al. (eds.): Cellular Adhesion. Molecular Definition to Therapeutic Potential. pp- 259-290. Plenum Press, New York, 1994.

51.   Ellington, A.D. and Conrad, R. (1995). In Biotechnology Annual Review, Vol 1. M.R. Elgewely, ed. (Elsevier Science Pub), pp. 185-214.

52.   Asseline, V. et al. (1994), PNAS USA **81**, 3297-3301.

53.   Nielsen, P.E. et al. (1991), Science **254**, 1497-1500.

54.   Shepherd, R.K. et al. (1996), Circ. Res. **78**, 627-634.

55.   Webb, T.E. et al. (1996), Mol. Pharmacol. **50**, 258-265.

56.   Farrell, D. H., and D. D. Cunningham. **1986.** *Proc. Natl. Acad. Sci.* USA **83**: 6858-62

57. Craig, P. A., S. T. Olson, and J. D. Shore. **1989.**
J. Biol. Chem. **264**: 5452-61.

58. Abelson, J.N., ed., (1996): Meth. Enzymol. **267**.
Combinatorial Chemistry. Academic Press, San Diego 1996.

59. Cortese, R, ed. (1996): Combinatorial Libraries.
Synthesis, Screening and Application Potential. Walter
de Gruyter. Berlin 1996.

60. Wu, A.M. (1996), Nat.Biotech. **14**, 429-431.

61. Wadhwa, M.S. (1995), Bioconj. Chem. **6**, 283-291.

62. Toole, B.P. **1990.** *Curr. Opin. Cell Biol.* **2**, 839-844

63. Umezawa, F. and Eto, Y. (1988) *Biochem. Biophys.
Res. Commun.* **153**, 1038-1044

64. Spellerberg, B., Prasad, S., Cabellos, C.,
Burroughs, M., Cahill, P. and Tuomanen, E. (1995) *J.
Exp. Med.* **182**, 1037-1043.

65. Shi, F. L., C. Bailey, A. W. Malick, and K. L.
Audus. 1993. *Pharmaceutical Research* **10**: 282-288

66. Lee, R. J., and P. S. Low. **1995.** *Biochim. Biophys.
Acta - Biomembranes* **1233**: 134-144

67. Said, H. M., D. Hollander, and R. Mohammadkhani.
1993. *Biochim.Biophys.Acta* **1148**: 263-268.

Ref.: Passe, T.J., D.A. Bluemke and S.S. Siegelman.
1997. *Radiology* **203**: 593-600.

References to the preceding tables

A.   Auerbach, W., and R. Auerbach. **1994**. "Angiogenesis
inhibition: a review". *Pharmac. Ther.* **63**: 265-311.

B.   Barinaga, M. **1997**. "Designing Therapies That Target
Tumor Blood Vessels". *Science* **275** (Jan. 24): 482-484.

C.   Folkman, J., P. B. Weisz, M. M. Joullié, W. W. Li,
and W. R. Ewing. **1989**. "Control of Angiogenesis With
Synthetic Heparin Substitutes". *Science* **243**: 1490-1493.

D.   Fox, S. B., and A. L. Harris. **1997**. "Markers of
tumor angiogenesis: Clinical applications in prognosis
and anti-angiogenic therapy". *Investigational New Drugs*
15 (1): 15-28.

E.   Gastl, G., T. Hermann, M. Steurer, J. Zmija, E.
Gunsilius, C. Unger, and A. Kraft. **May 1997**.
"Angiogenesis as a target for tumor treatment". *Oncology*
54 (3): 177-184.

F.   Griffioen, A. W., M. J. H. Coenen, C. A. Damen, S.
M. M. Hellwig, D. H. J. Vanweering, W. Vooys, G. H.
Blijham, and G. Groenewegen. **1 August 1997**. "CD44 is
involved in tumor angiogenesis; an activation antigen on
human endothelial cells". *Blood* **90** (3): 1150-1159.

G.   Hlatky, L., P. Hahnfeldt, and C. N. Coleman. **1996**.
"Vacular endothelial growth factor: environmental
controls and effects in angiogenesis". *Brit. J. Cancer*
74 (**Suppl. XXVII**): S151-S156.

H.   Maragoudakis, M. E., E. Pipili-Synethos, E.
Sakkoula, D. Panagiotopoulos, N. Craniti, and J. M.
Matsoukas. **1996**. "Inhibition of TRAP-induced
angiogenesis by the tripeptide Phe-Pro- Arg, a

thrombin-receptor-derived peptide analogue". *Letters in Peptide Science* **3**: 227-232.

I.   Nguyen, M. **1997**. "Angiogenic factors as tumor markers". *Investigational New Drugs* **15** (1): 29-37.

J.   Ono, M., H. Izumi, S. Yoshida, D. Gtot, S. Jimi, N. Kawahara, T. Shono, S. Ushiro, M. Ryuto, K. Kohno, Y. Sato, and M. Kuwano. 1996. "Angiogenesis as a new target for cancer treatment". *Cancer Chemoter. Pharmacol.* **38** **(Suppl.)**: S78-S82.

K.   Passe, T. J., D. A. Bluemke, and S. S. Siegelman. **June 1997**. "Tumor angiogenesis: Tutorial on implications for imaging". *Radiology* **203** (3): 593-600.

L.   Saclarides, T. J. **February 1997**. "Angiogenesis in colorectal cancer". *Surgical Clinics of North America* **77** (1): 253.

M.   Sage, E. H. **May 1997**. "Pieces of eight: Bioactive fragments of extracellular proteins as regulators of angiogenesis". *Trends in Cell Biology* **7** (5): 182-186.

N.   Sagi-Assif, O., A. Traister, B. Z. Katz, R. Anavi, M. Eskenazy, and I. P. Witz. 1996. "TNFα and anti-Fas antibodies regulate Ly-6E.1 expression by tumor cells: A possible link between angiogenesis and Ly-6E.1". *Immunology Letters* **54**: 207-213.

O.   Strawn, L. M., G. McMahon, H. App, R. Schreck, W. R. Kuchler, M. P. Longhi, T. H. Hui, C. Tang, A. Levitzki, A. Gazit, I. Chen, G. Keri, L. Orfi, W. Risau, I. Flamme, A. Ullirch, K. P. Hirth, and L. K. Shawyer. 1996. "Flk-1 as a Target for Tumor Growth Inhibition". *Cancer Res.* **56**: 3340-3545.

P.   Stromblad, S., and D. A. Cheresh. **December 1996.**
"Cell adhesion and angiogenesis". *Trends in Cell Biology*
6 (12): 462-468.

5   Q.   Stromblad, S., and D. A. Cheresh. **November 1996.**
"Integrins, angiogenesis and vascular cell survival".
Chemistry & Biology 3 (11): 881-885.

R.   Volpert, O., D. Jackson, N. Bouck, and D. I. H.
10   Linzer. **September 1996.** "The insulin-like growth factor
II/mannose 6-phosphate receptor is required for
proliferin-induced angiogenesis". *Endocrinology* 137 (9):
3871-3876.

15   S.   Yoshida, O. M., T. Shono, H. Izumi, T. Ishibashi,
H. Suzuki, and M. Kuwano. **1997.** "Involvement of
Interleukin-8, Vascular Endothelial Growth Factor, and
Basic Fibroblast Growth Factor in Tumor Necrosis Factor
Alpha-Dependent Angiogenesis". *Mol. Cell. Biol.* 17:
20   4015-4023.

T.   Zimrin, A. B., M. S. Pepper, G. A. McMahon, F.
Nguyen, R. Montesano, and T. Maciag. **1996.** "An Antisense
Oligonucleotide to the Notch Ligand Jagged Enhances
25   Fibroblast Growth Factor-induced Angiogenesis <in
vitro>". *J. Biol. Chem.* 271 (Dec. 20): 32499-3502.

U.   Albini, A., R. Soldi, D. Giunciuglio, E. Giraudo,
R. Benelli, R. Primo, D. Noonan, M. Salio, G. Camussi,
30   W. Rockl, and F. Bussolino. **1996.** "The angiogenesis
induced by HIV-1 Tat protein is mediated by the
Flk-1/KDR receptor on vacular endothelial cells". *Nature
Medicine* 2 (12 (Dec.)): 1371-1374.

35   V.   Ferrara, N. **1996.** "The biology of vascular
endothelial growth factor". in *Molecular, Cellular and
Clinical Aspects of Angiogenesis*, ed. M. E.

- 77 -

Maragoudakis. New York: Plenum Press.

X   Jackson, R. L., S. J. busch, and A. J. Cardin.
1991. "Glycosaminoglycans: Molecular Properties, Protein
Interactions, and Role in Physiological Processes".
Physiological Reviews **71** (2): 481-435.

Y.   Kinsella, M. G., C. K. Tsoi, H. T. Jarvelainen, and
T. N. Wight. **1997.** "Selective expression and processing
of biglycan during migration of bovine aortic
endothelial cells - The role of endogenous basic
fibroblast growth factor". *Journal of Biological
Chemistry* **272:** 318-325.

z.   Folkman, J. **1996.** Tumor angiogenesis and tissue
factor. *Nature Medicine* **2,** 167-8

ZA.   Relf, M., S. LeJeune, P.A. Scott, S. Fox, K. Smith,
R. Leek, A. Moghaddam, R. Whitehouse, R. Bicknell and
A:L. Harris. **1997.** "Expression of the angiogenic factors
vascular endothelial cell growth factor, acidic and
basic fibroblast growth factor, tumor growth factor
beta-1, platelet-derived endothelial cell growth factor,
placenta growth factor and pleiotrophin in human primary
breast cancer and its relation to angiogenesis". *Cancer
Res.* **57,** 963-9.

ZB.   Carmeliet, P., L. Moons, M. Dewerchin, N. Mackman,
T. Luther, G. Breier, V. Ploplis, M. Müller, A. Nagy, E.
Plow, R. Gerard, T. Edgington, W. Risau, D. Collen.
1997. *Ann, N.Y. Acad. Sci.* **811,** 191-206.

ZC.   Van Hinsbergh, P. Koolwijk, R. Haanemaijer. **1997.**
"Role of fibrin and plasminogen activators in repair-
associated angiogenesis: in vitro studies with human
endothelial cells" *EXS* **79,** 391-411.

The following non-limitative examples serve to
illustrate the invention. Confirmation of the
microparticulate nature of products is performed using
microscopy as described in WO-A-9607434. Ultrasonic
5   transmission measurements may be made using a broadband
transducer to indicate suspensions of products giving an
increased sound beam attenuation compared to a standard.
Flow cytometric analysis of products can be used to
confirm attachment of macromolecules thereto. The
10   ability of targeted agents to bind specifically to cells
expressing a target may be studied by microscopy and/or
using a flow chamber containing immobilised cells, for
example employing a population of cells expressing the
target structure and a further population of cells not
15   expressing the target. Radioactive or fluorescent
enzyme-labelled streptavidin/avidin may used to analyse
biotin attachment.

Example 1 - Gas-filled microbubbles encapsulated with
phosphatidylserine, phosphatidylcholine and biotin-
amidocaproate-PEG$_{3400}$-Ala-cholesterol

a) Synthesis of Z-Ala-cholesterol (3-O-(carbobenzyloxy-
L-alanyl)cholesterol)

Cholesterol (4mmol), Z-alanine (5 mmol) and
dimethylaminopyridine (4 mmol) were dissolved in
dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and
dicyclohexylcarbodiimide was added. The reaction mixture
was stirred at ambient temperature overnight.
Dicyclohexylurea was filtered off and the solvent was
rotary evaporated. The residue was taken up in
chloroform, undissolved dicyclohexylurea was filtered
off and the solvent was removed by rotary evaporation.
The residue was placed on a column of silica gel, and Z-
Ala-cholesterol was eluted with toluene/petroleum ether
(20:2) followed by toluene/diethyl ether (20:2). The
fractions containing the title compound were combined
and the solvent was removed by rotary evaporation. The
structure of the product was confirmed by NMR.

b) Synthesis of Ala-cholesterol (3-O-(L-alanyl)-
cholesterol)
Z-Ala-cholesterol (0.48 mmol) is placed in
tetrahydrofuran (20 ml) and glacial acetic acid (3 ml)
and hydrogenated in the presence of 5 % palladium on
charcoal for 2 hours. The reaction mixture is filtered
and concentrated in vacuo.

c) Synthesis of Boc-NH-PEG$_{3400}$-Ala-cholesterol

Ala-cholesterol is added to a solution of Boc-NH-PEG$_{3400}$-
SC (t-butyl carbamate poly(ethylene glycol)-
succinimidyl carbonate) (Shearwater) in chloroform,
followed by triethylamine. The suspension is stirred at

41 °C for 10 minutes. The crude product is purified by
chromatography.

d)   Synthesis of H_2N-PEG$_{3400}$-Ala-cholesterol

Boc-NH-PEG$_{3400}$-Ala-cholesterol is stirred in 4 M
hydrochloric acid in dioxane for 2.5 hours at ambient
temperature. The solvent is removed by rotary
evaporation and the residue is taken up in chloroform
and washed with water. The organic phase is rotary
evaporated to dryness. The crude product may be
purified by chromatography.

e)   Synthesis of biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol

A solution of biotinamidocaproate N-hydroxysuccinimide
ester in tetrahydrofuran is added to H_2N-PEG$_{3400}$-Ala-
cholesterol dissolved in tetrahydrofuran and 0.1 M
sodium phosphate buffer having a pH of 7.5 (2 ml). The
reaction mixture is heated to 30 °C and the reaction is
followed to completion by TLC, whereafter the solvent is
evaporated.

f)   Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol

To a mixture (5 mg) of phosphatidylserine and
phosphatidylcholine (in total 90-99.9mol%) and
biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol (10-0.1mol%)
is added 5% propyleneglycol-glycerol in water (1 ml).
The dispersion is heated to not more than 80 °C for 5
minutes and then cooled to ambient temperature. The
dispersion (0.8 ml) is then transferred to a vial (1 ml)
and the head space is flushed with perfluorobutane. The
vial is shaken in a cap-mixer for 45 seconds, whereafter

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the sample is put on a roller table. After
centrifugation the infranatant is exchanged with water
and the washing is repeated.

g) Alternative preparation of gas-filled microbubbles
encapsulated with phosphatidylserine,
phosphatidylcholine and biotinamidocaproate-PEG$_{3400}$-Ala-
cholesterol

To a mixture (5 mg) of phosphatidylserine and
phosphatidylcholine is added 5% propyleneglycol-glycerol
in water (1 ml). The dispersion is heated to not more
than 80 °C for 5 minutes and then cooled to ambient
temperature. The dispersion (0.8 ml) is then
transferred to a vial (1 ml) and the head space is
flushed with perfluorobutane. The vial is shaken in a
cap-mixer for 45 seconds, whereafter the sample is put
on a roller table. After centrifugation the infranatant
is exchanged with water. Biotinamidocaproate-PEG$_{3400}$-Ala-
cholesterol dissolved in water is added the washed
microbubbles, which are placed on a roller table for
several hours. The washing procedure is repeated
following incorporation of the biotinamidocaproate-
PEG$_{3400}$-Ala-cholesterol into the microbubble membranes.

Example 2 - Gas-containing microparticles comprising
phosphatidylserine, phosphatidylcholine, biotin-
amidocaproate-PEG$_{3400}$-Ala-Cholesterol and drug-cholesterol

a) Synthesis of drug-cholesterol

Cholesterol (4mmol), a drug having an acid group (see
Example 2(b) for a list of cholesterol-derivatised
drugs) and dimethylaminopyridine (4 mmol) are dissolved
in dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and
dicyclohexylcarbodiimide is added. The reaction mixture
is stirred at ambient temperature overnight.

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Dicyclohexylurea is filtered off and the solvent is rotary evaporated. The title compound is purified by chromatography.

5 b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine, biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol and drug-cholesterol

10 To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9mol%) and biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol (prepared as in Example 1) and drug-cholesterol (in total 10-0.1mol%) is added 5% propyleneglycol-glycerol in water (1 ml).

15 The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds whereafter

20 the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated.

Example 3 - Biotin attached to gas-filled microbubbles

25

Biotin may be attached to microbubbles in many different ways, e.g. in a similar way to that described by Corley, P. and Loughrey, H.C. in (1994) *Biochim. Biophys. Acta* **1195**, 149-156. The resulting bubbles are analysed by

30 flow cytometry, e.g. by employing fluorescent streptavidin to detect attachment of biotin to the bubbles. Alternatively radioactive or enzyme-labelled streptavidin/avidin is used to analyse biotin attachment.

35

Example 4 - Gas-filled microbubbles encapsulated with 1.2-distearoyl-sn-Glycero-3-[Phospo-L-Serine] and

<u>biotin-DPPE</u>

1.2-distearoyl-sn-Glycero-3-[Phospo-L-Serine] (Avanti
lot# 180PS-12, 22.6 mg) was added 4% propylenglycol-
5   glycerol in water (4 ml). The dispersion was heated, to
not more than 80 °C for five minutes, and then cooled to
ambient temperature. An aqueous dispersion of biotin-
DPPE (Pierce lot# 96092472, 1.5 mg) in 4%
propylenglycol-glycerol (1 ml) was added and the sample
10   was put on a roller table for 1-2 hours. The suspension
was filled on vials and head spaces were flushed with
perfluorobutane. The vials were shaken for 45 seconds
whereafter they were put on a roller table. After
centrifugation for seven minutes the infranatant was
15   exchanged with water and the washing was repeated two
times.

Normal Phase HPLC with an Evaporative Light Scattering
Detector confirmed that the membranes of the
20   microbubbles contained 4 mol% biotin-DPPE. The mean
particle diameter of the microbubbles was 4 μm measured
by Coulter Counter. Ultrasound transmission measurements
using a 3.5 MHz broadband transducer showed that a
particle dispersion of < 2 mg/ml gave a sound beam
25   attenuation higher than 5 dB/cm.

<u>Example 5 - Gas-filled microbubbles encapsulated with
phosphatidylserine and biotinylated oligonucleotide non-</u>
30   <u>covalently bound to streptavidin-Succ-PEG-DSPE</u>

<u>a)   Synthesis of Succ-PEG$_{3400}$-DSPE</u>

NH$_2$-PEG$_{3400}$-DSPE (prepared as in Preparation 1) is
35   carboxylated using succinic anhydride, e.g. by a similar
method to that described by Nayar, R. and Schroit, A.J.
in *Biochemistry* (1985) 24, 5967-5971.

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b) <u>Preparation of gas-filled microbubbles encapsulated</u>
<u>with phosphatidylserine and Succ-PEG₃₄₀₀-DSPE</u>

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
5   and Succ-PEG$_{3400}$-DSPE (10-0.1 mol%) is added 5%
propyleneglycol-glycerol in water (1 ml). The
dispersion is heated to not more than 80 °C for 5
minutes and then cooled to ambient temperature. The
dispersion (0.8 ml) is transferred to a vial (1 ml) and
10 the head space is flushed with perfluorobutane. The
vial is shaken in a cap-mixer for 45 seconds, whereafter
the sample is put on a roller table. After
centrifugation the infranatant is exchanged with water
and the washing is repeated. Alternatively the
15 microbubbles may be prepared as described in Preparation
1(f).

c) <u>Coupling of streptavidin to gas-filled microbubbles</u>
<u>encapsulated with phosphatidylserine and Succ-PEG$_{3400}$-DSPE</u>

20

Streptavidin is covalently bound to succ-PEG$_{3400}$-DSPE in
the microbubbles by standard coupling methods using a
water-soluble carbodiimide. The sample is placed on a
roller table during the reaction. After centrifugation
25 the infranatant is exchanged with water and the washing
is repeated. The functionality of the attached
streptavidin is analysed by binding, e.g. to
fluorescently labeled biotin, biotinylated antibodies
(detected with a fluorescently labeled secondary
30 antibody) or biotinylated and fluorescence- or
radioactively-labeled oligonucleotides. Analysis is
performed by fluorescence microscopy or scintillation
counting.

35 d) <u>Preparation of gas-filled microbubbles encapsulated</u>
<u>with phosphatidylserine and a biotinylated</u>
<u>oligonucleotide non-covalently bound to streptavidin-</u>

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succ-PEG-DSPE

succ-PEG₃₄₀₀-DSPE

Microbubbles from (c) above are incubated in a solution
containing a biotinylated oligonucleotide. The
5   oligonucleotide-coated bubbles are washed as described
above. Binding of the oligonucleotide to the bubbles is
detected e.g. by using fluorescent-labeled
oligonucleotides for attachment to the bubbles, or by
hybridising the attached oligonucleotide to a labeled
10   (fluorescence or radioactivity) complementary
oligonucleotide. The functionality of the
oligonucleotide-carrying microbubbles is analysed, e.g.
by hybridising the bubbles with immobilized DNA-
containing sequences complementary to the attached
15   oligonucleotide. As examples, an oligonucleotide
complementary to ribosomal DNA (of which there are many
copies per haploid genome) and an oligonucleotide
complementary to an oncogene (e.g. ras of which there is
one copy per haploid genome) are used.

20

Example 6 - The peptide FNFRLKAGQKIRFGAAAWEPPRARI
attached to gas-filled microbubbles encapsulated with
phosphatidylserine

25   The peptide FNFRLKAGQKIRFGAAAWEPPRARI, comprising
phosphatidylserine-binding and heparin-binding sections,
is synthesised. The peptide is added to preformed
phosphatidylserine-encapsulated perfluorobutane
microbubbles and thoroughly mixed.

30

Example 7 - Gas-filled microbubbles encapsulated with
phosphatidylserine and inactivated human thrombin-Succ-
PEG₃₄₀₀-DSPE

35   a) Inactivation of Human thrombin

Human thrombin is inactivated by incubation with a 20 %

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molar excess of D-Phe-L-Pro-L-Arg-chloromethyl ketone in
0.05 M HEPES buffer, pH 8.0, at 37 °C for 30 minutes.

b)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and Succ-PEG$_{3400}$-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
and Succ-PEG$_{3400}$-DSPE (10 - 0.1 mol%, prepared as in
Example 5(a)) is added 5% propyleneglycol-glycerol in
water (1 ml). The dispersion is heated to not more than
80 °C for 5 minutes and is then cooled to ambient
temperature. The dispersion (0.8 ml) is transferred to
a vial (1 ml) and the head space is flushed with
perfluorobutane. The vial is shaken in a cap-mixer for
45 seconds, whereafter the sample is put on a roller
table. After centrifugation the infranatant is
exchanged with water and the washing is repeated.
Alternatively the microbubbles may be prepared as
described in Preparation 1(f).

c)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and inactivated human thrombin-
Succ-PEG$_{3400}$-DSPE

Inactivated human thrombin is covalently bound to succ-
PEG$_{3400}$-DSPE in the microbubbles from (b) above by
standard coupling methods using a water-soluble
carbodiimide. The sample is placed on a roller table
during the reaction. After centrifugation the
infranatant is exchanged with water and the washing is
repeated.

Example 8 - Gas-containing microparticles comprising
polymer from ethylidene bis(16-hydroxyhexadecanoate) and
adipoyl chloride and biotin-amidocaproate-Ala covalently
attached to the polymer

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<u>a)   Synthesis of Z-Ala-polymer (3-O-(carbobenzyloxy-L-alanyl)-polymer)</u>

The polymer is prepared from ethylidene bis(16-
hydroxyhexadecanoate) and adipoyl chloride as described
in WO-A-9607434, and a polymer fraction with molecular
weight 10000 is purified using gel permeation
chromatography (GPC). 10 g of the material
(corresponding to 1 mmol OH groups), Z-alanine (5 mmol)
and dimethylaminopyridine (4 mmol) are dissolved in dry
dimethylformamide/tetrahydrofuran and
dicyclohexylcarbodiimide is then added. The reaction
mixture is stirred at ambient temperature overnight.
Dicyclohexylurea is filtered off and the solvent is
removed using rotary evaporation. The product is
purified by chromatography, fractions containing the
title compound are combined and the solvent is removed
using rotary evaporation. The structure of the product
is confirmed by NMR.

<u>b)   Synthesis of Ala-polymer (3-O-(L-alanyl)-polymer)</u>

Z-Ala-polymer (0.1 mmol) is stirred in
toluene/tetrahydrofuran and glacial acetic acid (15% of
the total volume) and hydrogenated in the presence of 5
% palladium on charcoal for 2 hours. The reaction
mixture is filtered and concentrated *in vacuo.*

<u>c)   Synthesis of biotinamidocaproate-Ala-polymer</u>

A solution of biotinamidocaproate N-hydroxysuccinimide
ester in tetrahydrofuran is added to
H₂N-Ala-polymer dissolved in a mixture of
tetrahydrofuran and dimethylformamide and 0.1 M sodium
phosphate buffer having a pH of 7.5. The reaction
mixture is heated to 30 °C and stirred vigorously; the
reaction is followed by TLC to completion. The solvent

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is evaporated and the crude product is used without
further purification.

d) Gas-containing particles comprising biotin-
amidocaproate-Ala-polymer and PEG 10000 methyl ether 16-
hexadecanoyloxyhexadecanoate

10 ml of a 5% w/w solution of biotin-amidocaproate-Ala-
polymer in (-)-camphene maintained at 60 °C is added to
30 ml of an 1% w/w aqueous solution of PEG 10000 methyl
ether 16-hexadecanoyloxyhexadecanoate (prepared as
described in WO-A-9607434) at the same temperature. The
mixture is emulsified using a rotor stator mixer (Ultra
Turax® T25) at a slow speed for several minutes, and
thereafter is frozen in a dry ice/methanol bath and
lyophilized for 48 hours, giving the *title product* as a
white powder.

e) Acoustic characterisation and microscopy of the
product

Confirmation of the microparticulate nature of the
product is performed using light microscopy as described
in WO-A-9607434. Ultrasonic transmission measurements
using a 3.5 MHz broadband transducer indicate that a
particle suspension of < 2 mg/ml gives a sound beam
attenuation of at least 5 dB/cm.

Example 9 - Functionalisation of of gas-filled albumin
microspheres (GAM) with biotin

A homogeneous suspension of GAM (6×10^8 particles/ml) in
5 mg/ml albumin was used, with all manipulations being
carried out at room temperature. Two 10 ml aliquots
were centrifuged (170 x g, 5 minutes) to promote
flotation of the microspheres and 8 ml of the underlying
infranatant was removed by careful suction and replaced

by an equal volume of air-saturated phosphate buffered
saline, the preparations being rotated for 15-20 minutes
to resuspend the microspheres. This procedure was
repeated twice, whereafter only negligible amounts of
free non-microsphere-associated albumin were assumed to
remain.

50 µl. of NHS-biotin (10 mM in dimethylsulphoxide) was
added to one of the aliquots (final concentration 50
µM); the other (control) aliquot received 50 µl of
dimethylsulphoxide. The tubes containing the samples
were rotated for 1 hour whereafter 20 µl portions of 50%
aqueous glutaraldehyde were added to each tube to
crosslink the microspheres. After rotation for another
hour the tubes were positioned vertically overnight to
allow flotation of the microspheres. The next day, the
suspensions were washed twice with phosphate buffered
saline containing 1 mg/ml human serum albumin (PBS/HSA)
and were resuspended in PBS/HSA after the last
centrifugation.

In order to determine the presence of microsphere-
associated biotin, streptavidin conjugated to
horseradish peroxidase (strep-HRP) was added to both
suspensions and the tubes were rotated for 1 hour to
allow for reaction. The microspheres were then washed
three times, resuspended in 100 mM citrate-phosphate
buffer (pH 5) containing 0.1 mg/ml phenylenediamine
dihydrochloride and 0.01% hydrogen peroxide, and rotated
for 10 minutes. Development of a yellow-green colour
was indicative of the presence of enzyme. The following
results were obtained:

Sample	Colour development
Biotinylated spheres + strp-HRP	2+

Control spheres + strp-HRP   +

This confirms that GAM were biotinylated.

5   Example 10 - Method of use

The agent from Example 7, comprising phosphatidylserine-
encapsulated microbubbles having inactivated human
thrombin-Succ-PEG$_{3400}$-DSPE incorporated into the
10   encapsulating membrane is lyophilised from 0.01 M
phosphate buffer, pH 7.4. The product is redispersed in
sterile water and injected intravenously into a patient
with suspected venous thrombosis in a leg vein. The leg
is examined by standard ultrasound techniques. The
15   thrombus is located by increased contrast as compared
with surrounding tissue.
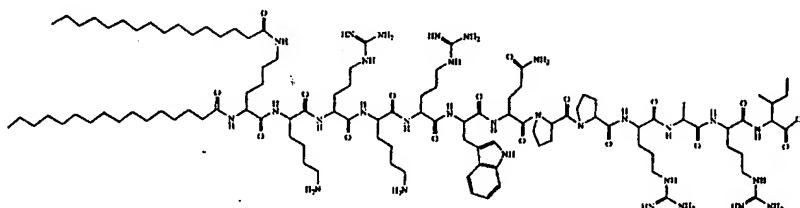
-91-

<u>Example 11 - Adhesion of poly-L-lysine-coated</u>
<u>phosphatidylserine-encapsulated microbubbles to</u>
<u>endothelial cells</u>

5   Poly-L-lysine (8 mg, Sigma P-1274 lot no. 14H5546)
      having a molecular weight of 115 kDa was dissolved in
      water (400 µl). Freshly redispersed microbubbles of
      phosphatidylserine-encapsulated perfluorobutane (40 µl)
      were incubated in either water (400 µl) or the
10   poly-L-lysine solution for 15 minutes at room
      temperature. Zeta potential measurements confirmed that
      the poly-L-lysine-coated microbubbles were positively
      charged while the uncoated bubbles were negatively
      charged. A cell adhesion study using human endothelial
15   cells grown in culture dishes (Type CRL 1730) was
      performed with the above-described microbubbles, the
      uncoated microbubbles being used as a control.
      Microscopy of the endothelial cells after incubation
      showed a much increased number of poly-L-lysine-coated
20   microbubbles adhering to endothelial cells in comparison
      to the uncoated microbubbles.

<u>Example 12 - Preparation and biological evaluation of</u>
<u>gas-containing microbubbles of DSPS 'doped' with a</u>
25 <u>lipopeptide consisting of a heparin sulphate binding</u>
<u>peptide (KRKR) and a fibronectin peptide (WQPPRARI).</u>

<u>a) Synthesis of a lipopeptide consisting of a heparin</u>
<u>sulphate binding peptide (KRKR) and fibronectin peptide</u>
30 <u>(WQPPRARI).</u>

35

The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Fmoc-Ile-Wang resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids and palmitic acid were
5   preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and
side-chain protecting groups was carried out in TFA
containing 5% phenol , 5% EDT, 5% anisole and 5% H_2O
for 2 hours giving a crude product yield of 150 mg.
10   Purification by preparative HPLC (Vydac 218TP1022
column) of a 40 mg aliquot of crude material was carried
out using a gradient of 70 to 100 % B over 40 min (A=
0.1 % TFA/water and B = MeOH) at a flow rate of 9
mL/min. After lyophilization 16 mg of pure material was
15   obtained (Analytical HPLC; Gradient, 70-100%B where B=
MeOH, A= 0.01% TFA/water: column - vydac 218TP54:
Detection - UV 260 and fluorescence, Ex_{280}, Em_{350} -
product retention time = 19.44 min). Further product
characterization was carried out using MALDI mass
20   spectrometry; expected, M+H at 2198, found, at 2199.

b) Preparation of gas-containing microbubbles of DSPS
'doped' with a lipopeptide consisting of a heparin
sulphate binding peptide (KRKR) and fibronectin peptide
25   (WQPPRARI).

DSPS (Avanti, 4.5 mg) and lipopeptide from a) (0.5 mg)
were weighed into each of 2 vials and 0.8 mL of a
solution of 1.4% propylene glycol/2.4% glycerol was
30   added to each vial. The mixture was warmed to 80°C for 5
minutes (vials shaken during warming). The samples were
cooled to room temperature and the head space flushed
with perfluorobutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles rolled overnight.
35   Bubbles were washed several times with deionised water
and analysed by Coulter counter (Size: 1-3 micron (87
%), 3-5 micron (11.5%)) and acoustic attenuation

(frequency max att.: 3.5 MHz). The microbubbles were
stable at 120 mm Hg.
MALDI mass spectral analysis was used to confirm
incorporation into DSPS microbubbles as follows; ca.
0.05-0.1 mL of microbubble suspension was transferred to
a clean vial and 0.05-0.1 mL methanol added. The
suspension was sonicated for 30 s and the solution
analysed by MALDI MS. Positive mode gave M+H at 2200,
expected for lipopeptide, 2198.

c) In vitro study of gas-containing microbubbles of DSPS
'doped' with a lipopeptide consisting of a heparin
sulphate binding peptide (KRKR) and fibronectin peptide
(WQPPRARI): binding to endothelial cells under flow
conditions

The human endothelial cell line ECV 304, derived from a
normal umbilical cord (ATCC CRL-1998) was cultured in
260 mL Nunc culture flasks (chutney 153732) in RPMI 1640
medium (Bio Whittaker) to which L-Glutamine 200 mM,
Penicillin/ Streptomycin (10.000 U/mL and 10.000 mcg/mL)
and 10% Fetal Bovine Serum (Hyclone Lot no. AFE 5183)
were added.
The cells were subcultured with a split ratio of 1:5 to
1:7 when reaching confluence.
Cover-glasses, 22mm in diameter (BDH, Cat no.
406/0189/40) were sterilised and placed on the bottom of
12 well culture plates (Costar) before cells in 0,5 mL
complete medium with serum was added on top.
When the cells reached confluence the coverslips were
placed in a custom made flow-chamber. The chamber
consists of a groove carved into a glass plate upon
which the cover slip with cells was placed with the
cells facing the groove forming a flow channel.
Ultrasound microbubbles from section b) were passed from
a reservoir held at 37 degree Celsius through the flow
chamber and back to the reservoir using a peristaltic

pump. The flow rate was adjusted to simulate
physiological relevant shear rates. The flow chamber was
placed under a microscope and the interaction between
the microspheres and cells viewed directly. A camera
5   mounted on the microscope was connected to a colour
video printer and a monitor.

A gradual accumulation of the microbubbles on the cells
took place which was dependant on the flow rate. By
increasing the flow rate the cells started to become
10   detached from the coverslip, the microbubbles were still
bound to the cells. Control bubbles not carrying the
vector did not adhere to the endothelial cells and
disappeared from the cells under minimal flow
conditions.

15

d) In vivo experiment in dog

Case 1)

20   A 22 kg mongrel dog was anaesthetized with pentobarbital
and mechanically ventilated. The chest was opened by a
midline sternotomy, the anterior pericardium was
removed, and a 30 mm gelled silicone rubber spacer was
inserted between the heart and a P5-3 transducer of an
25   ATL HDI-3000 ultrasound scanner. The scanner was set for
intermittent short axis imaging once in each end-systole
by delayed EGC triggering.

A net volume of 2 mL of microbubbles from b) were
injected as a rapid intravenous bolus. 3 seconds later,
30   the imaged right ventricle was seen to contain contrast
material, another 3 seconds later, the left ventricle
was also filled, and a transient attenuation shadow that
obscured the view of the posterior parts of the left
ventricle was observed. A substantial increase in
35   brightness of the myocardium was seen, also in the
portions of the heart distal to the left ventricle when
the attenuation shadow subsided.

After passage of the inital bolus, the ultrasound
scanner was set to continuous, high frame rate high
output power imaging, a procedure known to cause
destruction of utrasound contrast agent bubbles in the
imaged tissue regions. After a few seconds, the scanner
was adjusted back to its initial setting. The myocardium
was then darker, and closer to the baseline value.
Moving the imaged slice to a new position resulted in
re-appearance of contrast effects, moving the slice back
to the initial position again resulted in a tissue
brightness again close to baseline.

Case 2) [comparative]

A net volume of 2 mL microbubbles prepared in an
identical manner to b) above with the exception that no
lipopeptide was included in the preparation was
injected, using the same imaging procedure as above. The
myocardial echo enhancement was far less intense and of
shorter duration than observed in case 1. At the
completion of the left ventricular attenuation phase,
there was also almost complete loss of myocardial
contrast effects, and a myocardial echo increases in the
posterior part of the left ventricle as in case 1 was
not observed.

Example 13 - Targeted gas-containing microbubbles of
DSPS coated non-covalently with polylysine and a fusion
peptide comprising a PS binding component and a
Fibronectin peptide sequence
NH2.F.N.F.R.L.K.A.G.O.K.I.R.F.G.G.G.G.W.O.P.P.R.A.I.OH.

a) Synthesis of PS binding/Fibronectin fragment fusion
peptide
NH2.F.N.F.R.L.K.A.G.O.K.I.R.F.G.G.G.G.W.O.P.P.R.A.I.OH.

The peptide was synthesised on an ABI 433A automatic

peptide synthesiser starting with Fmoc-Ile-Wang resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids were preactivated using
HBTU before coupling.

5   The simultaneous removal of peptide from the resin and
side-chain protecting groups was carried out in TFA
containing 5% phenol , 5% EDT and 5% H_2O for 2 hours
giving a crude product yield of 302 mg. Purification by
preparative HPLC (Vydac 218TP1022 column) of a 25 mg

10   aliquot of crude material was carried out using a
gradient of 20 to 40 % B over 40 min (A= 0.1 % TFA/water
and B = 0.1 % TFA/acetonitrile) at a flow rate of 9
mL/min. After lyophilization 10 mg of pure material was
obtained (Analytical HPLC; Gradient, 20 to 50% B where

15   B= 0.1% TFA/acetonitrile, A= 0.01% TFA/water: column -
vydac 218TP54: Detection - UV 214 and 260 nm - product
retention time = 12.4 min). Further product
characterization was carried out using MALDI mass
spectrometry; expected, M+H at 2856, found, at 2866.

20

b) Preparation of microbubbles of DSPS coated non-
covalently with polylysine and the PS
binding/Fibronectin fragment fusion peptide
NH_2F.N.F.R.L.K.A.G.Q.K.I.R.F.G.G.G.W.Q.P.P.R.A.I.OH.

25

DSPS (5 mg, Avanti) was weighed into a clean vial along
with poly-L-lysine (Sigma, 0.2 mg) and peptide from a)
above (0.2 mg). To the vial was added 1.0 mL of a
solution of 1.4% propylene glycol/ 2.4% glycerol. The

30   mixture was warmed to 80°C for 5 minutes. The sample was
cooled to room temperature and the head space flushed
with perfluorobutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles centrifuged at 1000
rpm for 3 minutes.

35   Following extensive washing with water, PBS and water
the final solution was examined for polylysine and
peptide content using MALDI MS. No polypeptide material

was observed in the final wash solution.
Acetonitrile (0.5 mL) was then added and the
microbubbles destroyed by sonication. Analysis of the
resulting solution for polylysine and PS-
5   binding/fibronectin fusion peptide was then carried out
using MALDI MS. The results were as follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914, 1042, 1170	790, 919, 1048, 1177
DSPS-binding peptide	2856	2866

The spacer element contained within the PS
binding/Fibronectin fusion peptide (-GGG-) can also be
15   replaced with other spacers such as PEG_{2000} or poly
alanine (-AAA-). It is also envisaged that a form of
pre-targeting may be employed, whereby the DSPS
binding/Fibronectin fragment fusion peptide is firstly
allowed to associate with cells via the fibronectin
20   peptide binding. This is followed by administration of
PS microbubbles which then bind to the PS binding
peptide.

Example 14 - Gas containing microbubbles of DSPS
25   covalently modified with CD71 FITC-labelled anti-
transferrin receptor antibody and 'doped' with a
lipopeptide with affinity for endothelial cells.

This example is directed at the preparation of multiple
30   vector targeted ultrasound agents.

a) Synthesis of an endothelial cell binding lipopeptide:
2-n-hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-
Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH₂.

35

The lipopeptide shown below was synthesised on a ABI
433A automatic peptide synthesiser starting with a Rink

amide resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges.

5
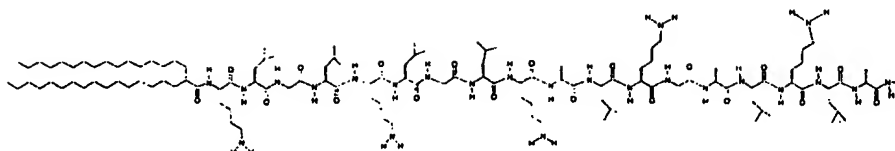
10   All amino acids and 2-n-hexadecylstearic acid were
      preactivated using HBTU before coupling. The
      simultaneous removal of peptide from the resin and side-
      chain protecting groups was carried out in TFA
      containing 5% EDT, and 5% H_2O for 2 hours giving a crude
15   product yield of 150 mg. Purification by preparative
      HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of
      crude material was carried out using a gradient of 90 to
      100 % B over 50 min (A= 0.1 % TFA/water and B = MeOH)
      at a flow rate of 9 mL/min. After lyophilization 10 mg
20   of pure material was obtained (Analytical HPLC;
      Gradient, 90-100%B where B= MeOH, A= 0.01% TFA/water:
      column - vydac 218TP54: Detection - UV 214 nm - product
      retention time = 23 min). Further product
      characterization was carried out using MALDI mass
25   spectrometry; expected, M+H at 2369, found, at 2373.

      b) Preparation of gas-containing microbubbles of DSPS
      'doped' with a endothelial cell binding lipopeptide and
      PE-PEG$_{2000}$-MAL

30
      DSPS (Avanti, 4.5 mg) and lipopeptide from a) (0.5 mg)
      along with PE-PEG$_{2000}$-Maleimide from example 2 (0.5 mg)
      were weighed into a clean vial and 1 mL of a solution of
      1.4% propylene glycol/2.4% glycerol added. The mixture
35   was warmed to 80°C for 5 minutes then filtered through a
      4.5 micron filter. The sample was cooled to room
      temperature and the head space flushed with

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perfluorobutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles washed three times
with distilled water.

5   c) Thiolation of FITC-labelled anti-transferrin receptor
antibody.

FITC labelled CD71 anti-transferrin receptor Ab (100 µg/
mL, Becton Dickinson), 0.7 mL, in PBS was modified with
10   Traut's reagent (0.9 mg, Pierce) at room temperature for
1 h. Excess reagent was separated from modified protein
on a NAP-5 column (Pharmacia).

d) Conjugation of thiolated FITC-labelled anti-
15   transferrin receptor antibody to gas-containing
microbubbles of DSPS 'doped' with an endothelial cell
binding lipopeptide and DSPE-PEG$_{2000}$-MAL

A 0.5 mL aliquot of the protein fraction (2 mL in total)
20   from c) above was added to the microbubbles from b) and
the conjugation reaction allowed to proceed for 10 min
on a roller table. Following centrifugation at 1000 rpm
for 3 min the protein solution was removed and the
conjugation repeated a further two times with 1 mL and
25   0.5 mL aliquots of protein solution respectively. The
bubbles were then washed four times in distilled water
and a sample analysed for the presence of antibody by
flow cytometry and microscopy. A fluorescent population
of >92% was observed.

30

Fig. 1 of the accompanying drawing represents the flow
cytometric comparison of negative control microbubbles of
DSPS (left curve) with bubbles conjugated with CD71 FITC-
labelled anti-transferrin antibody (filled curve, right),
35   showing that 92% of the population fluoresce.

- 100 -

Incorporation into the microbubbles of lipopeptide was confirmed by MALDI mass spectrometry as described in example 12 b).

5   Example 15 - Preparation of Transferrin/Avidin coated gas-filled microbubbles for targeted ultrasound imaging.

This example is directed to the preparation of microbubbles containing multiple protein vectors for
10 targeted ultrasound/therapy.

a) Synthesis of a thiol functionalised lipid molecule: Dipalmitoyl-Lys-Lys-Lys-Aca-Cys.OH

15

20
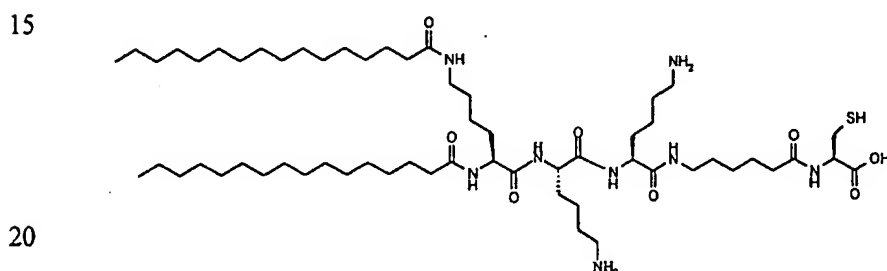
The lipid structure shown above was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Cys(Trt)-Wang resin (Novabiochem) on a 0.25 mmol scale
25 using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU coupling chemistry.
    The simultaneous removal of peptide from the resin and deprotection of side-chain protecting groups was carried
30 out in TFA containing 5% EDT, and 5% H_2O for 2 hours giving a crude product yield of 250 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of crude material was carried out using a gradient of 90 to 100 % B over 50 min (A= 0.1 % TFA/water and B =
35 MeOH) at a flow rate of 9 mL/min. After lyophilization 24 mg of pure material was obtained (Analytical HPLC; Gradient, 70-100%B where B= 0.1% TFA/ acetonitrile, A=

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0.01% TFA/water: column - vydac 218TP54: Detection - UV
214 nm-product retention time = 23 min). Further product
characterization was carried out using MALDI mass
spectrometry; expected, M+H at 1096, found, at 1099.

b) Preparation of gas-containing microbubbles of DSPS
'doped' with a thiol containing lipid structure:

DSPS (Avanti, 4.5 mg) and the lipid structure from a)
above (0.5 mg) were weighed into a clean vial and 0.8 mL
of a solution containing 1.4% propylene glycol/ 2.4%
glycerol in water added. The mixture was warmed to 80°C
for 5 minutes (vials shaken during warming) and filtered
while still hot through a 40 micron filter. The samples
were cooled to room temperature and the head space flushed
with perfluorobutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles placed on roller table
overnight. Bubbles were washed several times with
deionised water and analysed for thiol group incorporation
using Ellmans Reagent.

c) Modification of transferrin and avidin with
Fluorescein-NHS and Sulpho-SMPB.

To a mixture of 2 mg of transferrin (Holo, human, Alpha
Therapeutic Corp) and 2 mg of avidin (Sigma) in PBS (1 mL)
was added 0.5 mL DMSO solution containing 1 mg Sulpho-SMPB
(Pierce) and 0.5 mg Fluorescein-NHS (Pierce). The mixture
was stirred for 45 minutes at room temperature then passed
through a Sephadex 200 column using PBS as eluent. The
protein fraction was collected and stored at 4°C prior to
use.

d) Microbubble conjugation with modified
Transferrin/Avidin.

To the thiol containing microbubbles from b) was added 1

mL of the modified transferrin/avidin protein solution c).
After adjusting the pH of the solution to 9 the
conjugation reaction was allowed to proceed for 2 h at
room temperature. Following extensive washing with
5   deionised water the microbubbles were analysed by Coulter
counter (81% between 1 and 7 micron) and fluorescence
microscopy (highly fluorescent microbubbles were
observed).

10   <u>Example 16 - Preparation of functionalised gas-filled
microbubbles for targeted ultrasound imaging.</u>

This example is directed to the preparation of
microbubbles having a reactive group on the surface for
15   non-specific targeting, principally utilising disulphide
exchange reactions to effect binding to a multiplicity of
cellular targets.

DSPS (Avanti, 5.0 mg) and the thiol containing lipid
20   structure from example 15 a)(1.0 mg) were weighed into a
clean vial and 0.8 mL of a solution containing 1.4%
propylene glycol/ 2.4% glycerol in water added. The
mixture was warmed to 80°C for 5 minutes (vials shaken
during warming) and filtered while still hot through a 40
25   micron filter. The samples were cooled to room temperature
and the head space flushed with perfluorobutane gas. The
vials were shaken in a cap mixer for 45 s and the
microbubbles placed on roller table overnight. Bubbles
were washed several times with deionised water and
30   analysed for thiol group incorporation using Ellmans
Reagent.

<u>Example 17 - Gas-containing microbubbles of DSPS
35   comprising a lipopeptide for endothelial cell targeting
and a captopril containing molecule.</u>

This example is directed to the preparation of ultrasound
agents   for   combined   targeting   and   therapeutic
applications.

5   a)   Synthesis   of   a   lipopeptide   functionalised   with
       captopril:
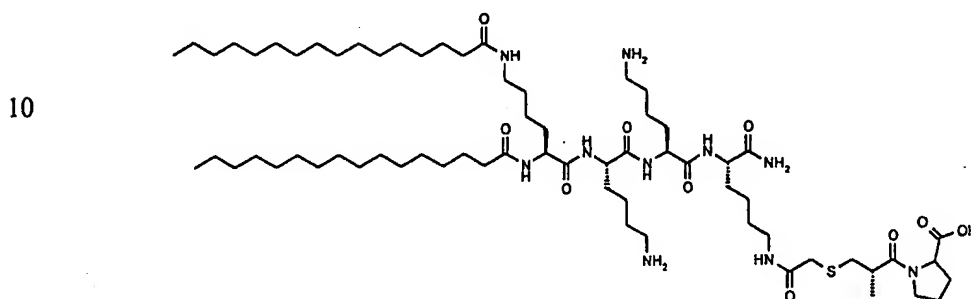
15

The structure shown above was synthesised using a manual
nitrogen bubbler apparatus starting with Fmoc protected
Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol
scale. All amino acids were purchased from Novabiochem
20   and palmitic acid from Fluka. Coupling was carried out
using standard TBTU/HOBt/DIEA protocols. Bromoacetic
acid was coupled through the side-chain of Lys as a
symmetrical anhydride using DIC preactivation. Captopril
(Sigma) dissolved in DMF was introduced on the solid-
25   phase using DBU as base.
.Simultaneous removal of the peptide from the resin and
deprotection of side-chain protecting groups was carried
out in TFA containing 5% EDT, 5% water and 5% ethyl
methyl sulphide for 2 h. An aliquot of 10 mg of the
30   crude material was purified by preparative liquid
chromatography (Vydac 218TP1022 column) using a gradient
of 70 to 100% B over 60 min (A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile) at a flow rate of 10 mL/min.
After lyophilization a yield of 2 mg of pure material
35   was obtained (analytical HPLC: gradient 70-100% B over
20 min, A = 0.1% TFA/water and B = 0.1%
TFA/acetonitrile; flow rate 1 mL/min; column Vydac

218TP54; detection UV 214 nm; retention time 26 min).
Further characterisation was carried out using MALDI
mass spectrometry, giving M+H at 1265 as expected.

5   b) Synthesis of a lipopeptide with affinity for
endothelial cells: Dipalmitoyl-Lys-Lys-Lys-Aca-Ile-Arg-
Arg-Val-Ala-Arg-Pro-Pro-Leu-NH₂

10

15   The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Rink amide resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids and palmitic acid were
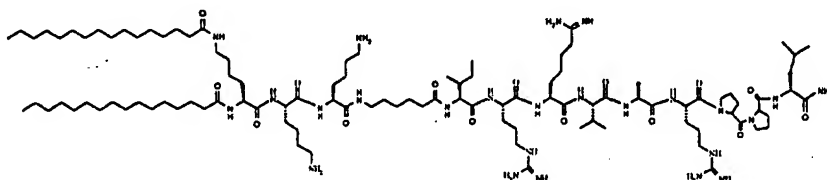preactivated using HBTU before coupling.

20   The simultaneous removal of peptide from the resin and
side-chain protecting groups was carried out in TFA
containing 5% phenol , 5% EDT and 5% H_2O for 2 hours
giving a crude product yield of 160 mg. Purification by
preparative HPLC (Vydac 218TP1022 column) of a 35 mg

25   aliquot of crude material was carried out using a
gradient of 70 to 100 % B over 40 min (A= 0.1 %
TFA/water and B = MeOH) at a flow rate of 9 mL/min.
After lyophilization 20 mg of pure material was obtained
(Analytical HPLC; Gradient, 70-100%B where B= MeOH, A=

30   0.01% TFA/water: column - vydac 218TP54: Detection - UV
214 and 260 nm - product retention time = 16 min).
Further product characterization was carried out using
MALDI mass spectrometry; expected, M+H at 2198, found,
at 2199.

35

c) Preparation of gas-containing microbubbles of DSPS
comprising a lipopeptide for endothelial cell targeting

and a captopril containing molecule for drug delivery

DSPS (Avanti, 4.5 mg), product from a) (0.5 mg) and
product from b) (0.5 mg) were weighed into a vial and
5   1.0 mL of a solution of 1.4% propylene glycol/ 2.4%
glycerol was added to each vial. The mixture was warmed
to 80°C for 5 minutes (vials shaken during warming). The
samples were cooled to room temperature and the head
space flushed with perfluorobutane gas. The vials were
10   firstly shaken in a cap mixer for 45 s then rolled for 1
h followed by extensive washing with deionised water. No
detectable levels of starting material was found in the
final wash solution as evidenced by MALDI MS.
MALDI mass spectral analysis was used to confirm
15   incorporation of the products from section a) and b)
into the microbubbles as described in example 12 b).

Example 18 - Preparation of gas-containing microbubbles
of DSPS loaded with a lipopeptide comprising a helical
20   peptide with affinity for cell membranes and the peptide
antibiotic polymixin B sulphate.

This example is directed at the preparation of targeted
microbubbles comprising multiple peptidic vectors having
25   a combined targeting and a therapeutic application.

a) Synthesis of a lipopeptide comprising a helical
peptide with affinity for cell
membranes:hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-Leu-Ala-
30   Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH$_2$.

Described in example 14 a).

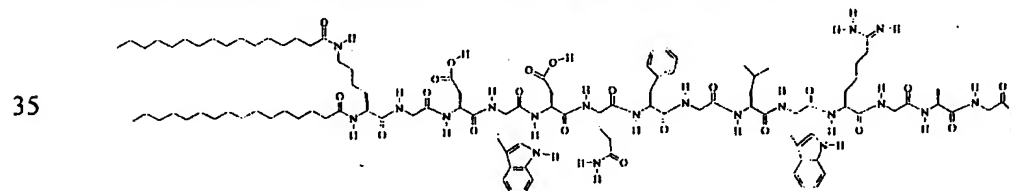b) - Preparation of multiple-specific gas-containing
35   microbubbles.

DSPS (Avanti, 5.0 mg), lipopeptide from a)(0.3 mg)and

polymixin B sulphate (Sigma,0.5 mg) was weighed into a
clean vial and 1.0 mL of a solution of 1.4% propylene
glycol/ 2.4% glycerol added. The mixture was sonicated
for 3-5 mins, warmed to 80°C for 5 minutes then

5   filtered through a 4.5 micron filter. The mixture was
cooled to room temperature and the head space flushed
with perfluorobutane gas. The vial was shaken in a cap
mixer for 45 s and the microbubbles centrifuged at 1000
rpm for 3 minutes. The microbubbles were washed in water

10   until no polymixin B sulphate or lipopeptide could be
detected in the infranatant by MALDI-MS. Microscopy
showed that the size distribution of the bubble
population was between 1-8 micron as desired.
To the washed bubbles (ca. 0.2 mL) was added methanol

15   (0.5 mL) and the mixture placed in a sonic bath for 2
min. The resulting clear solution, following analysis by
MALDI-MS, was found to contain both lipopeptide and
polymixin B sulphate (expected 1203, found 1207).

20   <u>Example 19 - Preparation of multiple-specific gas-
containing microbubbles of DSPS 'doped' with a
lipopeptide comprising a IL-1 receptor binding sequence
and modified with a branched structure containing the
drug methotrexate.</u>

25

This example is directed at the preparation of targeted
microbubbles comprising a non-bioactive vector for
targeting and a component for drug delivery.

30   <u>a) Synthesis of a lipopeptide comprising an interleukin-
1 receptor binding peptide: Dipalmitoyl-Lys-Gly-Asp-Trp-
Asp-Gln-Phe-Gly-Leu-Trp-Arg-Gly-Ala-Ala.OH</u>

35

The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Fmoc-Ala-Wang resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids and palmitic acid were
5 . preactivated using HBTU before coupling.
The simultaneous removal of lipopeptide from the resin
and side-chain protecting groups was carried out in TFA
containing 5% H_2O, 5% anisole, 5 % phenol and 5% EDT for
2 hours giving a crude product yield of 150 mg.
10 Purification by preparative HPLC (Vydac 218TP1022
column) of a 30 mg aliquot of crude material was carried
out using a gradient of 90 to 100 % B over 40 min (A=
0.1 % TFA/water and B = MeOH) at a flow rate of 9
mL/min. After lyophilization 4 mg of pure material was
15 obtained (Analytical HPLC; Gradient, 90-100%B over 20
min where B= MeOH, A= 0.01% TFA/water: column - vydac
218TP54: Detection - UV 214 nm; product retention time =
23 min). Further product characterization was carried
out using MALDI mass spectrometry; expected, M+H at
20 2083, found, at 2088.

b) Synthesis of a branched methotrexate core structure
containing a thiol moiety.

25

30
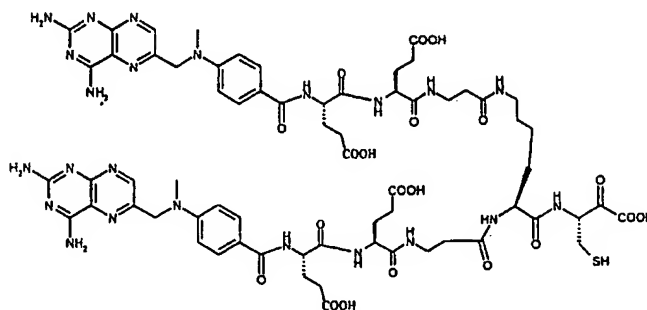
The methotrexate structure was synthesised on a ABI 433A
35 automatic peptide synthesiser starting with Fmoc-
Cys(Trt) Tentagel resin on a 0.1 mmol scale.
The simultaneous removal of product from the resin and

deprotection of protecting groups was carried out in TFA
containing 5% EDT and 5% H$_2$O for 2 hours giving a crude
product yield of 160 mg. Purification by preparative
HPLC (Vydac 218TP1022 column) of a 30 mg aliquot of
5   crude material was carried out using a gradient of 10 to
30 % B over 40 min (A= 0.1 % TFA/water and B = 0.1 %
TFA/acetonitrile) and a flow rate of 9 mL/min. After
lyophilization of the pure fractions 9 mg of pure
material was obtained (Analytical HPLC; Gradient, 5-50
10   %B where B = 0.1 % TFA/acetonitrile, A= 0.01% TFA/water:
column - vydac 218TP54: Detection - UV 214 nm - product
retention time = 9.5 min). Further product
characterization was carried out using MALDI mass
spectrometry; expected, M+H at 1523, found, 1523.

15

c)- Preparation of multiple-specific gas-containing
microbubbles.

DSPS (Avanti, 4.5 mg) and thiol containing lipopeptide
20   from example 15 a) (0.5 mg) and lipopeptide from a) (0.2
mg) above were weighed into a clean vial and 1.0 mL of a
solution of 1.4% propylene glycol/ 2.4% glycerol added.
The mixture was sonicated for 3-5 mins, warmed to 80°C
for 5 minutes then filtered through a 4.5 micron filter.
25   The mixture was cooled to room temperature and the head
space flushed with perfluorobutane gas. The vials were
shaken in a cap mixer for 45 s and the microbubbles
centrifuged at 1000 rpm for 3 minutes following which
the infranatant was discarded.

30

d) Conjugation of methotrexate branched structure to
thiolated microbubbles.

The methotrexate structure from b) above (0.5 mg) was
35   dissolved in PBS pH 8.0. The solution was then added to
the thiol containing bubbles from c) and disulphide bond
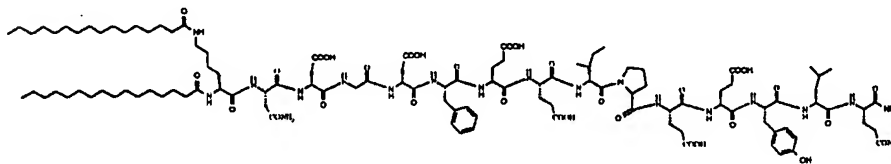formation allowed to proceed for 16 h. Following

extensive washing with PBS and water the bubbles were
analysed by microscopy and MALDI MS.

It is also considered relevant that the disulphide bond
5   linking the methotrexate structure to the microbubble
may be reduced in vivo liberating the free drug
molecule. This in combination with a tumour specific
vector is a drug delivery system. A physiologically
relevant reducing agent such as glutathione may be used
10   to bring about drug release.

Example 20 - Preparation of microbubbles encapsulated
with DSPS and functionalised with a thrombi-targeting
lipopeptide and the thrombolytic enzyme tissue
15   plasminogen activator.

This example is directed at the preparation of thrombus
targeted US agents comprising a therapeutic thromolytic
agent.
20
a) Synthesis of a lipopeptide with affinity for thrombi
(Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-
Glu-Tyr-Leu-Gln.NH₂).

25

30
The lipopeptide was synthesised on a ABI 433 A automatic
peptide synthesiser starting with Rink amide resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids and palmitic acid were
35   preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and
side-chain protecting groups was carried out in TFA

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containing 5% phenol, 5% EDT, 5% anisole and 5% H$_2$0 for
2 h giving a crude product yield of 80 mg. Purification
by preparative HPLC (Vydac 218TP1022 column) of a 20 mg
aliquot of the crude material was carried out. After
5 lyophilization 6 mg of pure material was obtained. The
product was characterized by MALDI mass spectrometry and
analytical HPLC.

b) Modification of tissue plasminogen activator with
10 Sulpho-SMPB.

A solution of 0.1 mL of ammonim carbonate buffer
containing 0.1 mg of t-PA (Sigma) was made up to 0.2 mL
by the addition of water. To this solution was added 0.4
15 mg of Sulpho-SMPB (Pierce) dissolved in 0.05 mL DMSO.
The protein solution was left standing at room
temperature for 45 min then purification carried out on
a Superdex 200 column. The product was eluted in PBS and
the modified protein fraction collected.

20

c) Preparation of microbubbles encapsulated with
DSPS/thrombi-binding lipopeptide and thiol containing
lipoeptide and conjugation of modified tissue
plasminogen activator.

25

DSPS (Avanti, 5.0 mg) was weighed into a clean vial
along with 0.5 mg of the lipopeptide from a) and 0.5 mg
of the thiol containing lipopeptide from example 15 a).
To this was added 1.0 mL of a solution of 1.4% propylene
30 glycol/ 2.4% glycerol and the mixture sonicated for 2
min then warmed to 80°C for 5 minutes. Immediately
following warming the solution was filtered through a 4
micron filter. The sample was cooled to room temperature
and the head space flushed with perfluorobutane gas. The
35 vials were shaken in a cap mixer for 45 s and the
microbubbles washed 2 times with deionised water. The
infranatant was discarded and replaced with a 1 mL

aliquot of the protein solution from b) above. The
conjugation reaction was allowed to proceed for 1 h. The
bubbles were centrifuged and infranatant exchanged with
a further 1 mL of protein solution. The incubation step
5   was repeated until all protein solution was used up. The
microbubbles were then washed extensively with water and
analysed by Coulter counter. The microbubbles were
tested in the flow chamber assay described in example 12
c). Microbubbles modified with protein were found to
10   bind in higher numbers than those comprising either
lipopeptide/DSPS or DSPS alone.

It is envisaged that the
targeting/therapeutic/ultrasound activities of these
15   microbubbles be evaluated in models of in vitro and in
vivo thrombogenisis.

Example 21 - Preparation of gas-containing microbubbles
20   encapsuled with DSPS comprising thiolated anti-CD34-MAL-
PEG₂₀₀₀-PE

a) Preparation of gas containing microbubbles encapsuled
with DSPS and PE-PEG 2000-Mal

25
DSPS (Avanti, 4,5 mg) and PE-PEG₂₀₀₀-maleimide from
example x (0,5 mg) were weighed into a clean vial and 1
mL of a solution of 1.4% propylene glyco/2.4% glycerol
added. The mixture was warmed to 80°C for 5 minutes then
30   filtered through a 4.5 micron filter. The sample was
cooled to room temperature and the head space flushed
with perfluorbutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles washed three times
with destilled water.

35
b) Thiolation of anti-CD34 antibodies.

- 112 -

To 0.3 mg of anti-CD34 antibody dissolved in 0.5 mL PBS
buffer pH7, was added 0.3 mg Traut's reagent and the
solution stirred at room temperature for 1 h. Excess
reagent was separated from the modified protein on a

5   NAP-5 column (Pharmacia).

c) Conjugation of thiolated anti-CD34 antibody to gas-
containing microbubbles encapsuled with DSPS and
comprising DSPE-PEG$_{2000}$-MAL

10

0.5mL of the thiolated antibody praparation from b) was
added to an aliqout of microbubbles from a) and the
conjugation reaction allowed to proceed for 30 min on a
roller table. Following centifugation at 2000 rpm for 5

15   min the infranatant was removed. The microbubbles were
washed a further three times with water.

d) Detection of the antibody encapsuled in the
microbubbles using a FITC-conjugated secondary antibody.

20

To the microbubbles suspension from c) was added 0.025
mL FITC-conjugated goat-anti-mouse antibody. The
mixture was incubated at room temperature (dark) for 30
min on a roller table then centrifugation at 2000 rpm

25   for 5 min. The infranatant was then removed and the
microbubbles washed a further three times with water.
Flow cytometric analysis of the microbubble suspension
showed that 98% of the population were fluorescent.

30   Example 22 - Preparation of gas-containing microbubbles
of DSPS loaded with a lipopeptide comprising a helical
peptide with affinity for cell membranes

This example is directed at the preparation of targeted

35   microbubbles comprising a non-bioactive peptidic vector
for targeting of cell membrane structures.

a) Synthesis of a lipopeptide comprising a helical
peptide with affinity for cell membranes

5
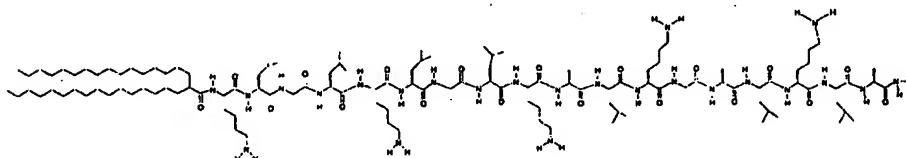
10   The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Rink amide resin
(Novabiochem) on a 0.2 mmol scale using 1mmol amino acid
cartridges. All amino acids and 2-n-hexadecylstearic
acid were preactivated using HBTU before coupling.

15   The simultaneous removal of lipopeptide from the resin
and side-chain protecting groups was carried out in TFA
containing 5% H_2O for 2 hours giving a crude product
yield of 520 mg. Purification by preparative HPLC (Vydac
218TP1022 column) of a 30 mg aliqout of crude material

20   was carried out using a gradient of 90 to 100 % B over
40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate
of 9 ml/min. After lyophilization 10 mg of pure material
was obtained (Analytical HPLC; Gradient, 90-100%B over
20 min where B= MeOH, A= 0.01% TFA/water: column - vydac

25   218TP54: Detection - UV 214 nm; product retention time =
23 min). Further product characterization was carried
out using MALDI mass spectrometry; expected, M+H at
2369, found, at 2375.

30   b) - Preparation of gas-containing microbubbles.

DSPS (Avanti, 4.5 mg) and lipopeptide from a)(0.5 mg)
was weighed into a clean vial and 1.0 ml of a solution
of 1.4% propylene glycol/ 2.4% glycerol added. The
35   mixture was sonicated for 3-5 mins, warmed to 80°C for 5
minutes then filtered through a 4.5 mm filter. The
mixture was cooled to room temperature and the head

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space flushed with perfluorobutane gas. The vials were
shaken in a cap mixer for 45 s and the microbubbles
centrifuged at 1000 rpm for 3 minutes. The bubbles were
washed in water until no lipopeptide could be detected
5   by MALDI-MS. Coulter counter, acoustic attenuation and
pressure stability studies were performed.
To an aliquot of the washed bubbles (ca. 0.2 mL) was
added methanol (0.5 mL) and the mixture placed in a
sonic bath for 2 min. The resulting clear solution,
10   following analysis by MALDI-MS, was found to contain the
lipopeptide. The microbubbles had similar
characteristics in vitro and in vivo as was found for
the microbubbles made in example 12.

15

Example 23 - Preparation of gas-containing microbubbles
of DSPS loaded with a lipopeptide comprising a non-
bioactive interleukin 1 receptor binding peptide

20   This example is directed at the preparation of targeted
microbubbles comprising a non-bioactive peptidic vector
for targeting at the IL-1 recptor which does not induce
signal tranduction or prevent IL-1 binding.

25   a) Synthesis of a lipopeptide comprising a non-bioactive
interleukin 1 receptor binding peptide

35   The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Fmoc-Ala-Wang resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino

acid cartridges. All amino acids and palmitic acid were
preactivated using HBTU before coupling.

The simultaneous removal of lipopeptide from the resin
and side-chain protecting groups was carried out in TFA
5   containing 5% H_2O, 5% anisole, 5 % phenol and 5% EDT for
2 hours giving a crude product yield of 150 mg.
Purification by preparative HPLC (Vydac 218TP1022
column) of a 30 mg aliqout of crude material was carried
out using a gradient of 90 to 100 % B over 40 min (A=
10   0.1 % TFA/water and B = MeOH) at a flow rate of 9
ml/min. After lyophilization 4 mg of pure material was
obtained (Analytical HPLC; Gradient, 90-100%B over 20
min where B= MeOH, A= 0.01% TFA/water: column - vydac
218TP54: Detection - UV 214 nm; product retention time =
15   23 min). Further product characterization was carried
out using MALDI mass spectrometry; expected, M+H at
2083, found, at 2088.

20   b) - Preparation of multiple-specific gas-containing
microbubbles.

DSPS (Avanti, 4.5 mg) and lipopeptide from a)(0.5 mg)
were weighed into a clean vial and 1.0 ml of a solution
25   of 1.4% propylene glycol/ 2.4% glycerol added. The
mixture was sonicated for 3-5 mins, warmed to 80°C for 5
minutes then filtered through a 4.5 micron filter. The
mixture was cooled to room temperature and the head
space flushed with perfluorobutane gas. The vials were
30   shaken in a cap mixer for 45 s and the microbubbles
centrifuged at 1000 rpm for 3 minutes. The bubbles were
washed in water until no lipopeptide could be detected
by MALDI-MS.

To the washed bubbles (ca. 0.2 mL) was added methanol
35   (0.5 mL) and the mixture placed in a sonic bath for 2
min. The resulting clear solution, following analysis by
MALDI-MS, was found to contain lipopeptide (expected

2083, found 2088).

Example 24 - Preparation of PFP containing microbubbles
of DSPC, DSPS and endothelial cell binding lipopeptide
for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPC:DSPS (3:1)
(5mg/ ml) in propyleneglycol/glycerol (4% in water) was
added 0.5 mg of the lipopeptide from example 17 b). The
mixture was heated to 80°C for 5 minutes and shaken.
The solution was then cooled to ambient temperature and
the headspace was flushed with perfluoropropane. The
vial was shaken on a Capmix (Espe Capmix) for 45 seconds
and placed on a roller table for 5 min. The sample was
centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes
and the infranatant removed and replaced with destilled
water. The headspace was again flushed with
perfluorobutane and the sample was kept on a table
roller until a homogenous appearance was obtained. The
washing procedure was repeated. The resulting ultrasound
contrast agent was confirmed by Coulter counter,
acoustic attenuation measurements and resistance to
external pressure.
The microbubbles were tested in the in vitro assay as
detailed in example 12. A gradual accumulation of
bubbles binding to the cells was observed.

Example 25 - Preparation of SF_6 containing microbubbles
of DSPC, DSPS and endothelial cell binding lipopeptide
for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPC:DSPS (3:1)
(5mg/ ml) in propyleneglycol/glycerol (4% in water) was
added 0.5 mg of the lipopeptide from example 17 b). The
mixture was heated to 80°C for 5 minutes and shaken.
The solution was then cooled to ambient temperature and

the headspace was flushed with SF$_6$ gas. The vial was
shaken on a Capmix (Espe Capmix) for 45 seconds and
placed on a roller table for 5 min. The sample was
centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes
5   and the infranatant removed and replaced with destilled
water. The headspace was again flushed with
perfluorobutane and the sample was kept on a table
roller until a homogenous appearance was obtained. The
washing procedure was repeated.
10   The resulting ultrasound contrast agent was confirmed by
Coulter counter, acoustic attenuation measurements and
resistance to external pressure.

Example 26 - Preparation of PFB containing microbubbles
15   of DSPG and endothelial cell binding lipopeptide for
targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in
propyleneglycol/glycerol (4% in water) was added 0.5 mg
20   of the lipopeptide from example 17 b). The mixture was
heated to 80°C for 5 minutes and shaken. The solution
was then cooled to ambient temperature and the headspace
was flushed with perfluorobutane. The vial was shaken on
a Capmix (Espe Capmix) for 45 seconds and placed on a
25   roller table for 5 min. The sample was centrifuged (Juan
MR 14.11) at 2000 rpm for 5 minutes and the infranatant
removed and replaced with destilled water. The headspace
was again flushed with perfluorobutane and the sample
was kept on a table roller until a homogenous appearance
30   was obtained. The washing procedure was repeated.
The resulting ultrasound contrast agent was confirmed by
Coulter counter, acoustic attenuation measurements and
resistance to external pressure.
The microbubbles were tested in the in vitro assay as
35   detailed in example 12. A gradual accumulation of
bubbles binding to the cells was observed.

Example 27 - Preparation of PFP containing microbubbles
of DSPG and endothelial cell binding lipopeptide for
targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in
propyleneglycol/glycerol (4% in water) was added 0.5 mg
of the lipopeptide from example 17 b. The mixture was
heated to 80°C for 5 minutes and shaken. The solution
was then cooled to ambient temperature and the headspace
was flushed with perfluoropropane. The vial was shaken
on a Capmix (Espe Capmix) for 45 seconds and placed on a
roller table for 5 min. The sample was centrifuged (Juan
MR 14.11) at 2000 rpm for 5 minutes and the infranatant
removed and replaced with destilled water. The headspace
was again flushed with perfluorobutane and the sample
was kept on a table roller until a homogenous appearance
was obtained. The washing procedure was repeated. The
resulting ultrasound contrast agent was confirmed by
Coulter counter, acoustic attenuation measurements and
resistance to external pressure.

The microbubbles were tested in the in vitro assay as
detailed in example 12. A gradual accumulation of
bubbles binding to the cells was observed.

Example 28 - Preparation of SF$_6$ containing microbubbles
of DSPG and endothelial cell binding lipopeptide for
targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in
propyleneglycol/glycerol (4% in water) was added 0.5 mg
of the lipopeptide from example 17 b). The mixture was
heated to 80°C for 5 minutes and shaken. The solution
was then cooled to ambient temperature and the headspace
was flushed with SF$_6$ gas. The vial was shaken on a
Capmix (Espe Capmix) for 45 seconds and placed on a
roller table for 5 min. The sample was centrifuged (Juan
MR 14.11) at 2000 rpm for 5 minutes and the infranatant

removed and replaced with destilled water. The headspace
was again flushed with perfluorobutane and the sample
was kept on a table roller until a homogenous appearance
was obtained. The washing procedure was repeated.

5   The resulting ultrasound contrast agent was confirmed by
Coulter counter, acoustic attenuation measurements and
resistance to external pressure.

10   Example 29 - Targeted gas-containing microbubbles of
DSPS coated non-covalently with polylysine

DSPS (5 mg, Avanti) was weighed into a clean vial along
with poly-L-lysine (Sigma, 0.2 mg). To the vial was
15   added 1.0 ml of a solution of 1.4% propylene glycol/
2.4% glycerol. The mixture was warmed to 80°C for 5
minutes. The sample was cooled to room temperature and
the head space flushed with perfluorobutane gas. The
vials were shaken in a cap mixer for 45 s and the
20   microbubbles centrifuged at 1000 rpm for 3 minutes.

Following extensive washing with water, PBS and water
the final solution was examined for polylysine content
using MALDI MS. No polypeptide material was observed in
25   the final wash solution.

Acetonitrile (0.5 ml) was then added and the
microbubbles sonicated until all bubbles had burst.
Analysis of the resulting solution for polylysine was
30   again carried out using MALDI MS. The results were as
follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914, 1042, 1170	790, 919, 1048,
		1177

35

Example 30 - Preparation of PFB gas-containing
microbubbles of DSPS doped with a thrombus binding
lipopeptide.

5   a) Synthesis of a lipopeptide with affinity for thrombi
(Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-
Glu-Tyr-Leu-Gln.NH₂).

10
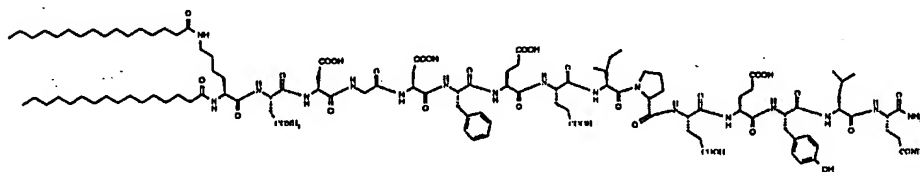
15   The lipopeptide was synthesised on a ABI 433 A automatic
peptide synthesiser starting with Rink amide resin
(Novabiochem) on a 0.1 mmol scale using 1 mmol amino
acid cartridges. All amino acids and palmitic acid were
preactivated using HBTU before coupling.

20   The simultaneous removal of peptide from the resin and
side-chain protecting groups was carried out in TFA
containing 5% phenol; 5% EDT, 5% anisole and 5% H₂O for
2 h giving a crude product yield of 80 mg. Purification
by preparative HPLC (Vydac 218TP1022 column) of a 20 mg

25   aliquot of the crude material was carried out. After
lyophilization 6 mg of pure material was obtained. The
product was characterized by MALDI mass spectrometry and
analytical HPLC.

30   b) Preparation of thromi-targeting ultrasound
microbubbles.

DSPS (Avanti, 4.5 mg) and lipopeptide from a) (1.0 mg)
were weighed into a vial and 0.8 ml of a solution of

35   1.4% propylene glycol/2.4% glycerol added. The mixture
was warmed to 80°C for 5 minutes then the sample
filtered through a 4 micron filter. After cooling to

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room temperature the head space was flushed with
perfluorobutane gas. The vials were shaken in a cap
mixer for 45 s and the microbubbles washed extensively
with deionised water. The bubbles were analysed by
microscopy Coulter counter. MALDI-MS was used to
confirm the presence of lipopeptide as previously
described.

Example 31- Gas-filled microbubbles of DSPS comprising a
lipopeptide consisting of a heparin sulphate binding
peptide (KRKR) and a fibronectin peptide (WQPPRARI) for
targeting and a lipopeptide containing atenolol for
therapeutic applications

a) Synthesis of a protected atenolol derivative suitable
for solid phase coupling

i) Synthesis of methyl 4-[(2,3-
epoxy)propoxy]phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
h. The reaction mixture was cooled, and excess
epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na$_2$SO$_4$). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz)
spectra were in accordance with the structure.

ii) Synthesis of methyl
4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceta

te

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)

5   and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na$_2$SO$_4$). Filtration
and concentration gave quantitative yield of a yellow

10 oil that was used in the next step without further
purification. The structure was verified by ^{1}H and ^{13}C
NMR analysis.

iii) Synthesis of

15 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti
c acid hydrochloride

A solution of methyl
4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceta

20 te (563 mg, 2.00 mmol) in 6M hydrochloric acid (15 ml)
was heated at 100 °C for 4h. The reaction mixture was
concentrated (rotavapor) and the residue was taken up in
water and lyophilised. ^{1}H and ^{13}C NMR spectra were in
accordance with the strucure and MALDI mass spectrometry

25 gave a M+H at 268 as expected.

iv) Synthesis of N-Boc-
4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti
c acid

30

A solution of the
4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti
c acid hydrochloride (2.0 mmol) in water (2 ml) was
added to a solution of sodium bicarbonate (0.60 g, 7.2

35 mmol) in water/dioxane (2:1, 15 ml). A solution of di-
tert-butyl dicarbonate (0.48 g, 2.2 mmol) in dioxane (5
ml) was added. Progress of the reaction was monitored

by TLC analysis .(silica, CHCl₃/MeOH/AcOH 85:10:5), and
portions of di-tert-butyl dicarbonate were added until
conversion was complete. The reaction mixture was poured
onto water saturated with potassium hydrogensulphate and
5   organic material was extracted into ethyl acetate. The
organic phase was washed with water and brine, dried
(Na₂SO₄) and filtered to give 0.6 g of crude material.
The product was purified by chromatography (silica,
CHCl₃/MeOH/AcOH 85:10:5). The solution was concentrated
10   and the residue was taken up in glacial acetic acid and
lyophilised. Yield 415 mg (56%), white solid. The
structure was confirmed by ¹H and ¹³C NMR analysis.

b)Synthesis of a lipopeptide functionalised with
15   atenolol
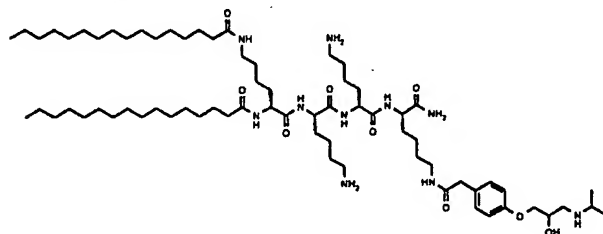
20

The structure shown above was synthesised using a manual
nitrogen bubbler apparatus starting with Fmoc protected
25   Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol
scale, using amino acids from Novabiochem, palmitic acid
from Fluka and the compound from a). Coupling was
carried out using standard TBTU/HOBt/DIEA protocols.
Simultaneous removal of the peptide from the resin and
30   deprotection of side-chain protecting groups was carried
out in TFA containing 5% EDT and 5% water for 2h. Crude
material was precipitated from ether and purified by
preparative liquid chromatography (Vydac 218TP1022
column) using a gradient of 70 to 100% B over 60 min (A
35   = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a
flow rate of 10 ml/min. After lyophilisation a yield of
38 mg of pure material was obtained (analytical HPLC:

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gradient 70-100% B over 20 min, A = 0.1% TFA/water and B
= 0.1% TFA/acetonitrile, flow rate 1 ml/min, column
Vydac 218TP54, detection UV 214 nm, retention time 25
min). Further characterisation was carried out using
5   MALDI mass spectrometry (ACH matrix), giving M+H at
1258, expected 1257.

c) Preparation of gas-filled microbubbles of DSPS
comprising a lipopeptide cosisting of a heparin sulphate
10   binding peptide (KRKR) and a fibronectin peptide
(WOPPRARI) and a lipopeptide containing atenolol for
therapeutic applications

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0
15   ml) was added to a mixture of DSPD (Avanti, 5.0 mg),
product from Example 12 a) (0.5 mg) and product from b)
(0.5 mg) in a vial. The mixture was sonicated for 5 min
and then heated at 80 °C for 5 min (vial was shaken
during warming). The solution was filtered and cooled.
20   Head space was flushed with perfluorobutane gas and the
vial was shaken in a cap mixer for 45s followed by
extensive washing with deionised water.

Incorporation of atenolol containing lipopeptide into
25   the bubbles was confirmed by MALDI-MS as follows. Ca 50
μl of microbubbles were transferred to a clean vial
containing ca 100 μl of 90% methanol. The mixture was
sonicated for 30s and analysed by MALDI-MS (ACH matrix),
giving two M+H peaks at 2202 and 1259, corresponding to
30   lipopeptide from Example 12 a) and to lipopeptide from
b), respectively.

The microbubbles were tested in the in vitro assay as
detailed in Example 12. A gradual accumulation of
35   bubbles binding to the cells was observed.

Example 32 - Gas-filled microbubbles encapsulated with
DSPS and a compound containing folic acid for diagnostic
applications

5   a) Synthesis of a lipopeptide containing folic acid
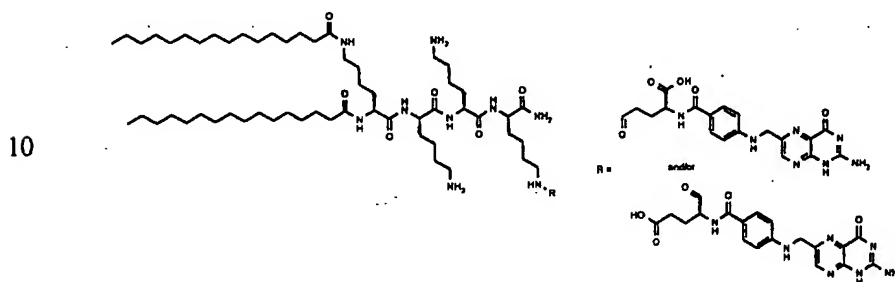
15   The structure shown above, with was synthesised using
     a manual nitrogen bubbler apparatus starting with Fmoc
     protected Rink Amide MBHA resin (Novabiochem) on a 0.125
     mmol scale, using amino acids from Novabiochem, palmitic
     acid from Fluka and folic acid from Acros. Coupling was
20   carried out using standard TBTU/HOBt/DIEA protocols.
     Simultaneous removal of the peptide from the resin and
     deprotection of side-chain protecting groups was carried
     out in TFA containing 5% EDT and 5% water for 2h. Crude
     material was precipitated from ether and analysed by
25   MALDI mass spectrometry, giving a M+H peak correspnding
     to the structure at 1435, expected 1430. The material
     was further characterised by analytical HPLC (column
     Vydac 218TP54, gradient 70-100% B over 20 min, A = 0.1%
     TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1.0
30   ml/min), giving a product peak with retention time 27
     min detected at UV 368 nm.

     b) Preparation of gas-containing microbubbles of DSPS
     comprising a lipopeptide containing folic acid

35

     A solution of 1.4% propylene glycol / 2.4% glycerol (1.0
     ml) was added t a mixture of DSPS (Avanti, 4.5 mg) and

product from a) (0.5 mg) in a vial. Dilute ammonia (to
pH 8) and DMSO (40 µl) were added and the mixture was
sonicated for 5 min and then heated at 80 °C for 5 min
(vial was shaken during warming). The solution was

5   filtered and cooled. Head space was flushed with
perfluorobutane gas and the vial was shaken in a cap
mixer for 45s followed by extensive washing with
deionised water.

10   Incorporation of structure from a) into the bubbles was
confirmed by MALDI-MS as follows. Ca 50 µl of
microbubbles were transferred to a clean vial containing
ca 100 µl of 90% methanol. The mixture was sonicated for
30s and analysed by MALDI-MS (ACH matrix), giving a M+H

15   peak at 1238 corresponding to structure from a).

The microbubbles were tested in the in vitro assay as
detailed in example 12. A gradual accumulation of
bubbles binding to the cells was observed.

20   ,

Example 33 - Gas-filled microbubbles of
phosphatidylserine comprising biotinamide-PEG-ß-Ala-
Cholesterol and a cholesteryl ester of chlorambucil for
25   diagnostic and therapeutic applications

a) Synthesis of cholesteryl N-Boc-ß-alaninate

DIC (510 µl) was added to a solution of Boc-ß-Ala-OH
30   (1.25 g, 6.60 mmol) in dichloromethane (15 ml) under an
inert atmosphere. The reaction mixture was stirred for
30 min and then transferred to a flask containing a
solution of cholesterol (1.16 g, 3.00 mmol) and DMAP
(367 mg, 3.00 mmol) in dichloromethane (15 ml). The
35   reaction mixture was stirred for 2 h and then poured
onto an aqeous solution of potassium hydrogensulphate.
Phases were separated and the aq phase was extracted

with chloroform. Combined organic phases were washed
with aq potassium hydrogensulphate and water and dried
(MgSO$_4$) - FEK023/031-01. After filtration and
evaporation the crude product was chromatographed
5   (silica, chloroform/methanol 99:1) to give 1.63 g (97%)
of white solid. The structure was confirmed by ^{1}H NMR
(500 Mhz).

b) Synthesis of cholesteryl ß-alaninate hydrochloride

10

A solution of compound from a) (279 mg, 0.500 mmol) in
1M hydrochloric acid in 1,4-dioxan (5 ml) was stirred at
room temperature for 4h. The reaction mixture was
concentrated to give a quantitative yield of cholesteryl
15   ß-alaninate hydrochloride. The structure was confirmed
by 1H NMR (500 MHz) analysis and by MALDI mass
spectrometry, giving a M+Na peak at 482, expected 481.

c) Biotin-PEG$_{3400}$-ß-Ala-Cholesterol

20

To a solution of cholesteryl ß-alaninate hydrochloride (15
mg, 0.03 mmol) in chloroform/wet methanol (2.6:1, 3 ml) was
added triethylamine (42 µl, 0.30 mmol). The mixture was
stirred for 10 minutes at room temperature and a solution
25   of biotin-PEG3400-NHS (100 mg, 0.03 mmol) in 1,4-dioxane
(1 ml) was added dropwise. After stirring at room
temperature for 3 hours, the mixture was evapourated to
dryness and the residue purified by flash chromatography
to give white crystals, yield ; 102 mg (89%). The
30   structure was verified by MALDI-MS and by NMR analysis.

d)Synthesis   of   cholesteryl   4-[4-[bis(2-
chloroethyl)amino]phenyl]butanoate

35

DIC (170 µl, 1.10 mmol) was added to a solution of
chlorambucil (Sigma, 669 mg, 2.20 mmol) in dry

dichloromethane (15 ml). The mixture was stirred at room
temperature for 0.5 h and added to a solution of
cholesterol (Aldrich, 387 mg, 1.00 mmol) and DMAP (122 mg,
1.00 mmol) in dichloromethane (10 ml). The reaction
5   mixture was stirred overnight and then poured onto 5%
sodium bicarbonate. The phases were separated and the
organic phase was washed with brine and dried (MgSO₄). The
solution was filtered and concentrated and the product was
purified by column chromatography (silica, chloroform) to
10   give 560 mg (83%) yield of colouless oil. The product was
characterised by MALDI mass spectrometry, giving M+H at
674 as expected. Further characterisation was carried out
using ¹H (500 MHz) and ¹³C (125 MHz) NMR analysis, giving
spectra in accordance with the structure.

15

e) Preparation of gas-filled microbubbles

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0
ml) was added to a mixture of DSPS (Avanti, 5 mg) and
20   product from c) (0.5 mg) and d) (0.5 mg) in a vial. The
mixture was sonicated for 5 min and then heated at 80 °C
for 5 min (vial was shaken during warming) and cooled.
Head space was flushed with perfluorobutane gas and the
vial was shaken in a cap mixer for 45s followed by
25   extensive washing with deionised water. MALDI mass
spectrometry showed no detectable level of compound from
c and d) in the final wash solution.

Incorporation of compounds from c) and d) into the bubbles
30   was confirmed by MALDI-MS as follows. Ca 50 µl of
microbubbles were transferred to a clean vial containing
ca 100 µl of 90% methanol. The mixture was sonicated for
30s and analysed by MALDI-MS (ACH-matrix), giving a M+H
peaks corresponding to compounds from c) and d).

35

Example 34 -Flotation of endothelial cells by micro

bubbles with vectors that specifically bind to the
endothelial cells

The human endothelial cell line ECV 304, derived from a
normal umbilical cord (ATCC CRL-1998) was cultures in Nunc
culture flasks (chutney 153732) in RPMI 1640 medium (Bio
Whitaker) to which L-Glutamine 200 mM,
Penicillin/Streptomycin (10.000 U/ml and 10.00 mcg/ml) and
10% Fetal Calf Serum (Hyclone Lot no AFE 5183) were added.
The cells were subcultured following trypsination with a
split ratio of 1:5 to 1:7 when reaching confluence.
2 mill. cells from trypsinated confluent cultures were
added to each set of 5 centrifuge tubes. Then control
microbubbles or bubbles carrying the vector including
WQPPARI (example 12), or the endothelial cell binding
peptide vector (example 14), were added at 2, 4, 6 ,8 or
10 mill bubbles per tube. The cells at the bottom of the
tubes after centrifugation at 400 g for 5 minutes were
counted with a Coulter counter. It was found the 4 or more
microbubbles binding to a cell did bring the cells to top
of the fluid in the centrifugation tube. All cells were
floated by the endothelial cell binding peptide vector and
about 50 % with the WQPPARI vector.

Example 35 - Gene transfer by PFB gas-filled microbubbles

This example is directed at the preparation of targeted
microbubbles for gene transfer.

a) Preparation of DSPS lipopeptide bubbles/PFB gas, coated
with polyl-L-lysine

DSPS (4,5 mg) and lipopeptide from 17 b) (0.5 mg) were
weighed in two 2-ml vials. To each vial, 0.8 ml
propyleneglycol/glycerol (4%) in water was added. The
solution was heated at 80°C for 5 minutes and shaken. The

solution was then cooled to ambient temperature and the headspace flushed with perfluorobutane. The vials were shaken on a Capmix (Espe Capmix, 4450 oscillations/min) for 45 seconds and put on a roller table for 5 minutes.
5   The content of the vials were mixed and the sample washed by centrifugation at 2000 rpm for 5 minutes. The infranatant was removed and the same volume of distilled water added. The washing procedure was repeated once.

10   Poly-L-lysine HBr (Sigma, 20.6 mg) was dissolved in 2 mL water then an aliquot (0.4 mL) made up to 2 mL water. To 1.2 mL of the diluted poly-L-lysine solution was added 0.12 mL of the DSPS-lipopeptide bubble suspension. Following incubation excess polylysine was removed by
15   extensive washing with water.

b) Transfection of cells

Endothelial cells (ECV 304) were cultured in 6 well plates
20   to a uniform subconfluent layer. A transfection mixture consisting of 5 µg DNA (an Enhanced Green Fluorescent Protein vector from CLONTECH) and 50 µl of microbubble suspension from a) in RPMI medium at a final volume of 250 µl was prepared. The mixture was left standing for 15 min
25   at room temperature then 1 mL of complete RPMI medium added. The medium was removed from the cell culture dish, and the DNA-microbubble mixture added to the cells. The cells were incubated in a cell culture incubator (37 °C).
30

c) Ultrasonic treatment

After 15 minutes incubation, selected wells were exposed to continious wave ultrasound of 1 MHz, 0.5 W/cm^2, for 30
35   seconds.

d) Incubation and examination

The cells were further incubated in the cell culture
incubator (37 °C) for approximately 4 1/2 hours. The
medium containing DNA-microbubbles was then removed by
aspiration, and 2 ml complete RPMI medium was added. The
5   cells were incubated for 40-70 hours before examination.
Most of the medium was then removed, and the cells were
examined by fluorescence microscopy. The results were
compared to the results from control experiments were DNA
or DNA-polylysine were added to the cells.

10

Claims

1.   A targetable diagnostic and/or therapeutically active agent comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generating material, said reporter being coupled or linked to one or more vectors, characterised in that said vector or vectors are non-bioactive.

2.   An agent as claimed in claim 1 wherein the gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, a low molecular weight hydrocarbon, a ketone, an ester, a halogenated low molecular weight hydrocarbon or a mixture of any of the foregoing.

3.   An agent as claimed in claim 2 wherein the gas comprises a perfluorinated ketone, perfluorinated ether or perfluorocarbon.

4.   An agent as claimed in claim 2 wherein the gas comprises sulphur hexafluoride or a perfluoropropane, perfluorobutane or perfluoropentane.

5.   An agent as claimed in any of the preceding claims comprising gas microbubbles stabilised by a coalescence-resistant surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.

6.   An agent as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.

7.   An agent as claimed in claim 6 wherein at least 75% of the said surfactant material comprises phospholipid molecules individually bearing net overall charge.

8.   An agent as claimed in claim 7 wherein at least 75%
of the film-forming surfactant material comprises one or
more phospholipids selected from phosphatidylserines,
phosphatidylglycerols, phosphatidylinositols, phosphatidic
acids and cardiolipins.

9.   An agent as claimed in claim 8 wherein at least 80%
of said phospholipids comprise phosphatidylserines.

10.   An agent as claimed in any of the preceding claims
comprising a combination of bioactive vectors, the
biological activities of which are counterbalanced such
that the combination is non-bioactive.

11.   An agent as claimed in any of the preceding claims
wherein the vector or vectors are monomeric or oligomeric.

12.   An agent as claimed in any of claims 1 to 10 wherein
the vector or vectors comprises non-bioactive peptides.

13.   An agent as claimed in any of claims 1 to 10 wherein
the vector or vectors are selected from antibodies; cell
adhesion molecules; cell adhesion molecule receptors;
cytokines; growth factors; peptide hormones and pieces
thereof; non-bioactive binders of receptors for cell
adhesion molecules; oligonucleotides and modified
oligonucleotides; DNA-binding drugs; protease
substrates/inhibitors; molecules generated from
combinatorial libraries and proteins and peptides which
bind to glucosaminoglycan side chains.

14.   An agent as claimed in any of the preceding claims
wherein the vector or vectors have affinity for targets at
a level such that the agent interacts with but does not
fixedly bind to said targets.

15.   An agent as claimed in claim 14 wherein the vector or

vectors are selected from ligands for cell adhesion
proteins and cell adhesion proteins which have
corresponding ligands on endothelial cell surfaces.

5   16.   An agent as claimed in any of the preceding claims
       wherein the vector or vectors are sited such that they are
       not readily exposed to the target.

       17.   An agent as claimed in any of the preceding claims
10   wherein the vector is covalently or non-covalently coupled
       or linked to the reporter.

       18.   An agent as claimed in any one of claims 1 to 16
       wherein the vector is coupled or linked to the reporter by
15   means of electrostatic charge interaction.

       19.   An agent as claimed in any one of claims 1 to 16
       wherein the vector is coupled or linked to the reporter by
       means   of   avidin-biotin   and/or   streptavidin-biotin
20   interactions.

       20.   An agent as claimed in any of the preceding claims
       which further contains moieties which are radiocactive or
       are effective as X-ray contrast agents, light imaging
25   probes or spin labels.

       21.   An agent as claimed in any of the preceding claims
       further comprising a therapeutic compound.

30   22.   An agent as claimed in claim 21 wherein said
       therapeutic compound is an antineoplastic agent, blood
       product, biological response modifier, antifungal agent,
       hormone or hormone analogue, vitamin, enzyme, antiallergic
       agent, tissue factor inhibitor, platelet inhibitor,
35   coagulation protein target inhibitor, fibrin formation
       inhibitor, fibrinolysis promoter, antiangiogenic,
       circulatory drug, metabolic potentiator, antitubercular,

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antiviral, vasodilator, antibiotic, antiinflammatory, antiprotozoan, antirheumatic, narcotic, opiate, cardiac glycoside, neuromuscular blocker, sedative, local anaesthetic, general anaesthetic or genetic material.

23. An agent as claimed in claim 21 or claim 22 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups.

24. An agent as claimed in claim 21 or claim 22 wherein a lipophilic or lipophilically-derivatised therapeutic compound is linked to the reporter through hydrophobic interactions.

25. A combined formulation comprising:
    i)   a first administrable composition comprising a pre-targeting vector having affinity for a selected target; and
    ii) a second administrable composition comprising an agent as claimed in any of the preceding claims, said agent comprising a vector having affinity for said pre-targeting vector.

26. A combined formulation as claimed in claim 25 wherein said pre-targeting vector comprises a monoclonal antibody.

27. A combined formulation comprising:
    i)   a first administrable composition comprising an agent as claimed in any of claims 1 to 24, and
    ii) a second administrable composition comprising a substance capable of displacing or releasing said agent from its target.

28. A combined formulation comprising:
    i)   a first administrable composition comprising an agent as claimed in claim 23, and
    ii) a second administrable composition comprising a

reducing agent capable of reductively cleaving the disulphide groups coupling or linking the therapeutic compound and reporter in the agent of said first administrable composition.

29. A process for the preparation of a targetable diagnostic and/or therapeutically active agent as defined in claim 1 which comprises coupling or linking at least one non-bioactive vector to a reporter comprising gas-containing or gas-generating material.

30. A process as claimed in claim 29 wherein a therapeutic compound is also combined with the reporter.

31. Use of an agent as claimed in any of claims 1 to 24 as a targetable ultrasound contrast agent.

32. A method of generating enhanced images of a human or non-human animal body which comprises administering to said body an agent as claimed in any of claims 1 to 24 and generating an ultrasound, magnetic resonance, X-ray, radiographic or light image of at least a part of said body.

33. A method as claimed in claim 32 which comprises the steps:
    i) administering to said body a pre-targeting vector having affinity for a selected target; and thereafter
    ii) administering an agent as claimed in any of claims 1 to 24, said agent comprising a vector having affinity for said pre-targeting vector.

34. A method as claimed in claim 33 wherein said pre-targeting vector comprises a monoclonal antibody.

35. A method as claimed in claim 32 which comprises the

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steps:

     i)   administering to said body an agent as claimed in any of claims 1 to 24; and thereafter

     ii)   administering a substance capable of displacing or releasing said agent from its target.

36. A method as claimed in any of claims 32 to 35 wherein said agent further comprises a therapeutic compound.

37. A method as claimed in claim 36 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups, and a composition comprising a reducing agent capable of reductively cleaving said disulphide groups is subsequently administered.

38. A method for *in vitro* investigation of targeting by an agent as defined in any of claims 1 to 24 wherein cells expressing a target are fixedly positioned in a flow chamber, a suspension of said agent in a carrier liquid is passed through said chamber, and binding of said agent to said cells is examined.

39. A method as claimed in claim 38 wherein the flow rate of carrier liquid is controlled to simulate shear rates encountered *in vivo*.
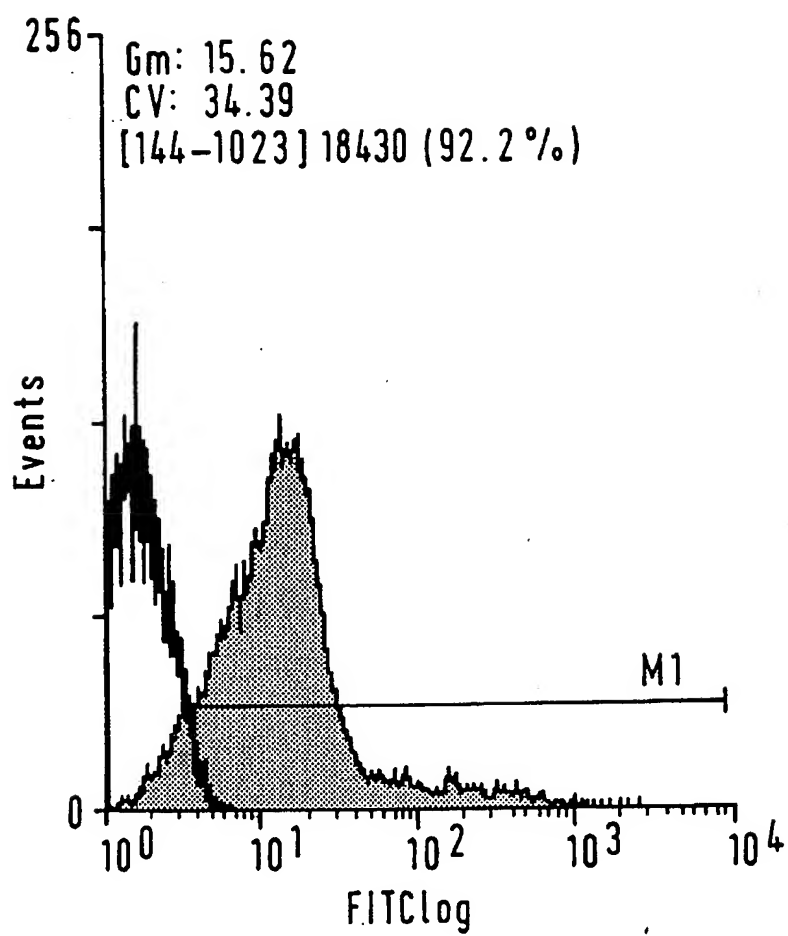
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FIG.1.